



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/31, C07K 14/27, A01H 1/00, 3/00	A3	(11) International Publication Number: WO 98/54214 (43) International Publication Date: 3 December 1998 (03.12.98)
(21) International Application Number: PCT/US98/10874 (22) International Filing Date: 28 May 1998 (28.05.98) (30) Priority Data: 60/048,109 30 May 1997 (30.05.97) US (71) Applicants: CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US). EDEN BIOSCIENCE CORPORATION [US/US]; 11816 North Creek Parkway N., Bothell, WA 98011-8205 (US). (72) Inventors: LABY, Ronald, J.; 1411 Haddon Street, Houston, TX 77006 (US). WEI, Zhong-Min; 8230 125th Court, Kirkland, WA 98034 (US). BEER, Steven, V.; 211 Hudson Street, Ithaca, NY 14850 (US). (74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> (88) Date of publication of the international search report: 4 March 1999 (04.03.99)
(54) Title: HYPERSENSITIVE RESPONSE ELICITOR FRAGMENTS AND USES THEREOF (57) Abstract <p>The present invention is directed to isolated fragments of an <i>Erwinia</i> hypersensitive response elicitor protein or polypeptide which fragments elicit a hypersensitive response in plants. Also disclosed are isolated DNA molecules which encode the <i>Erwinia</i> hypersensitive response eliciting fragment. Isolated fragments of hypersensitive response elicitor proteins or polypeptides, which elicit a hypersensitive response, and the isolated DNA molecules that encode them can be used to impart disease resistance to plants, to enhance plant growth, and/or to control insects on plants. This can be achieved by applying the hypersensitive response eliciting fragments in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a DNA molecule encoding a hypersensitive response eliciting fragment can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China			PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

HYPERSENSITIVE RESPONSE ELICITOR FRAGMENTS AND USES THEREOF

This application claims benefit of U.S. Patent Application Serial
5 No. 60/048,109.

FIELD OF THE INVENTION

The present invention relates to fragments of a hypersensitive response elicitor
10 which fragments elicit a hypersensitive response and uses thereof.

BACKGROUND OF THE INVENTION

Interactions between bacterial pathogens and their plant hosts generally fall
15 into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial
growth, symptom development, and disease development in the host plant; and (2)
incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular
type of incompatible interaction occurring, without progressive disease symptoms.
During compatible interactions on host plants, bacterial populations increase
20 dramatically and progressive symptoms occur. During incompatible interactions,
bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated
with the active defense of plants against many pathogens (Kiraly, Z., "Defenses
Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant Disease: An
25 Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed. Academic Press New
York (1980); Klement, Z., "Hypersensitivity," pages 149-177 in: Phytopathogenic
Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic Press, New York
(1982)). The hypersensitive response elicited by bacteria is readily observed as a
tissue collapse if high concentrations ($\geq 10^7$ cells/ml) of a limited host-range pathogen
30 like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of
nonhost plants (necrosis occurs only in isolated plant cells at lower levels of
inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic
Pseudomonads," Nature 199:299-300; Klement, et al., "Hypersensitive Reaction
Induced by Phytopathogenic Bacteria in the Tobacco Leaf," Phytopathology 54:474-

- 2 -

477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," Phytopathology 64:885-890 (1974); Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., "*hrp* Genes of Phytopathogenic Bacteria," pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangl, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," Trends Microbiol. 1:175-180 (1993)). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), Wei, Z.-H., et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a

- 3 -

New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994)).

5 The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive
10 response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp
15 Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-53 (1994)). However, *P. solanacearum popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated,
20 cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "Erwinia chrysanthemi Harpin_{Ech}: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996)); *Erwinia*
25 *stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

30 The present invention seeks to identify fragments of hypersensitive response elicitor proteins or polypeptides, which fragments elicit a hypersensitive response, and uses of such fragments.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated fragment of an *Erwinia*
5 hypersensitive response elicitor protein or polypeptide where the fragment elicits a hypersensitive response in plants. Also disclosed are isolated DNA molecules which encode such fragments.

The fragments of hypersensitive response elicitors can be used to impart disease resistance to plants, to enhance plant growth, and/or to control insects. This
10 involves applying the fragments in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

As an alternative to applying the fragments to plants or plant seeds in order to impart disease resistance, to enhance plant growth, and/or to control insects on plants,
15 transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a fragment of a hypersensitive response elicitor protein or polypeptide which fragments elicit a hypersensitive response in plants and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, and/or to control
20 insects in the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the DNA molecule encoding such a fragment can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, and/or to control insects on plants or plants grown from the plant seeds.

25

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a deletion and proteolysis analysis for the *Erwinia amylovora* hypersensitive response elicitor (i.e. harpin). A is the name of the harpin fragment. B
30 is the length of the fragment in amino acid residues. C indicates whether detectable protein is produced. D states whether there is hypersensitive response (i.e., HR) eliciting activity. The solid line indicates that there are additional amino acids which are not harpin encoded, while the dashed line indicates the portion of the harpin that is

- 5 -

deleted. The numbers above the fragments in the box represent the amino acid residue present at the end of a given fragment; residue #1 is the N-terminus, and residue #403 is the C-terminus.

Figure 2 is a Western blot illustrating specific secretion of harpin_{Ea}, but not harpin_{Ea}C31. Lane A, Ea273(pGP1-2) CFEP; lane B, Ea273(pGP1-2)(pCPP1104) CFEP; lane C, *E. coli* DH5 α (pCPP1107) CFEP harpin size standard; lane D, BioRad low range molecular weight markers; lane E, Ea273(pGP1-2) supernatant; lane F, Ea273(pGP1 2)(pCPP1104) supernatant. The blot was probed with an anti-harpin_{Ea} polyclonal antibody.

Figure 3 is an HR assay on tobacco leaf infiltrated as follows: (1) A, harpin_{Ea} + raspberry IF; (2) B, harpin_{Ea} + apple IF; (3) C, harpin_{Ea} + tobacco IF; (4) D, harpin_{Ea} + endoproteinase Glu-C; (5) E, harpin_{Ea} + trypsin; (6) F, harpin_{Ea}; (7) G, tobacco IF; (8) H, endoproteinase Glu-C; (9) I, trypsin; and (10) J, harpin_{Ea}. IF refers to intracellular fluids.

Figure 4 shows the digestion of harpin with endoproteinase Glu-C. Lane A is harpin; Lane B is harpin + endoproteinase Glu-C; Lane C is BioRad low range molecular weight markers.

Figure 5A shows the proteolysis of harpin. Coomassie blue stained polyacrylamide gel was loaded as follows: A, BioRad low range molecular weight markers; B, IF-apple; C, IF-raspberry; D, IF-tobacco; E, harpin_{Ea}; F, harpin_{Ea} + IF-apple; G, harpin_{Ea} + IF-raspberry; H, harpin_{Ea} + IF-tobacco.

Figure 5B shows a Coomassie Blue stained polyacrylamide gel loaded as follows: A, IF-tobacco; B, IF-tobacco + harpin_{Ea}; C, harpin_{Ea}; D, BioRad low range molecular weight markers; E, IF-tobacco + harpin_{Ea} + PMSF. HR-eliciting activity of the sample following proteolysis is denoted below the gel.

Figure 5C depicts whether proteolytic activity is present in IF from all plants tested. Intercellular fluid harvested from several plants was analyzed by PAGE in a gel containing 0.1% copolymerized gelatin. After washing to remove SDS and incubation to allow proteolysis of gelatin, the gels were stained to demonstrate the presence of gelatinolytic activity. A, IF-apple; B, IF-tobacco; C, IF-cotoneaster; D, BioRad mw; E, endoproteinase Glu-C; and F, ground leaf extract-tobacco.

- 6 -

Figure 6 shows the refractionation of elicitor-active peptides following proteolysis of harpin_{Ea} by tobacco IF. Absorbance was measured at 210 nm. Peak 1 contains peptides P91 and P95; peak 2 contains peptides P65 and P69.

Figure 7 shows the predicted proteolytic cleavage sites within harpin of several tested proteinases, and the effect of these cleavages on activity of active harpin fragments. Residues potentially important for HR-eliciting activity, based on the loss of activity following further cleavage, are indicated by upward-pointing arrows at bottom.

Figure 8 shows the similarities near N-termini among harpins of *Erwinia* spp. Underlined residues are present (identical or similar) in at least four out of the five proteins examined. Nine out of the first 26 residues are conserved in this manner.

Figures 9 A-B show Kyte-Doolittle hydropathy plots of bacterial HR-eliciting proteins. Ea, *E. amylovora* EA321; Est, *E. stewartii* DC283; Ech, *E. chrysanthemi* AC4150; Ecc, *E. carotovora* subsp. *carotovora*; Rs, *R. solanacearum*; Pss, *P. syringae* pv. *syringae*.

Figure 10 shows truncated proteins of the hypersensitive response elicitor protein or polypeptide.

Figure 11 shows a list of synthesized oligonucleotide primers for construction of truncated harpin proteins. N represents the N-terminus (5' region), and C represents the C-terminus (3' region). The primers correspond to the indicated sequence identification numbers for the present application: N1 (SEQ. ID. No. 1), N76 (SEQ. ID. No. 2), N99 (SEQ. ID. No. 3), N105 (SEQ. ID. No. 4), N110 (SEQ. ID. No. 5), N137 (SEQ. ID. No. 6), N150 (SEQ. ID. No. 7), N169 (SEQ. ID. No. 8), N210 (SEQ. ID. No. 9), N267 (SEQ. ID. No. 10), N343 (SEQ. ID. No. 11), C75 (SEQ. ID. No. 12), C104 (SEQ. ID. No. 13), C168 (SEQ. ID. No. 14), C180 (SEQ. ID. No. 15), C204 (SEQ. ID. No. 16), C209 (SEQ. ID. No. 17), C266 (SEQ. ID. No. 18), C342 (SEQ. ID. No. 19), and C403 (SEQ. ID. No. 20).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to isolated fragments of a hypersensitive response elicitor protein or polypeptide where the fragments elicit a hypersensitive response in plants. Also disclosed are DNA molecules encoding such fragments as

An example of a fungal source of a hypersensitive response elicitor protein or polypeptide is *Phytophthora*. Suitable species of *Phytophthora* include *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, and *Phytophthora citrophthora*.

The hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 21 as follows:

20	Met	Gln	Ile	Thr	Ile	Lys	Ala	His	Ile	Gly	Gly	Asp	Leu	Gly	Val	Ser	
	1				5				10				15				
	Gly	Leu	Gly	Ala	Gln	Gly	Leu	Lys	Gly	Leu	Asn	Ser	Ala	Ala	Ser	Ser	
				20					25					30			
25	Leu	Gly	Ser	Ser	Val	Asp	Lys	Leu	Ser	Ser	Thr	Ile	Asp	Lys	Leu	Thr	
		35					40						45				
	Ser	Ala	Leu	Thr	Ser	Met	Met	Phe	Gly	Gly	Ala	Leu	Ala	Gln	Gly	Leu	
		50					55					60					
	Gly	Ala	Ser	Ser	Lys	Gly	Leu	Gly	Met	Ser	Asn	Gln	Leu	Gly	Gln	Ser	
	65					70					75					80	
30	Phe	Gly	Asn	Gly	Ala	Gln	Gly	Ala	Ser	Asn	Leu	Leu	Ser	Val	Pro	Lys	
					85					90					95		
	Ser	Gly	Gly	Asp	Ala	Leu	Ser	Lys	Met	Phe	Asp	Lys	Ala	Leu	Asp	Asp	
				100					105					110			
	Leu	Leu	Gly	His	Asp	Thr	Val	Thr	Lys	Leu	Thr	Asn	Gln	Ser	Asn	Gln	
35			115					120					125				

- 8 -

	Leu	Ala	Asn	Ser	Met	Leu	Asn	Ala	Ser	Gln	Met	Thr	Gln	Gly	Asn	Met
	130					135						140				
	Asn	Ala	Phe	Gly	Ser	Gly	Val	Asn	Asn	Ala	Leu	Ser	Ser	Ile	Leu	Gly
	145					150					155					160
5	Asn	Gly	Leu	Gly	Gln	Ser	Met	Ser	Gly	Phe	Ser	Gln	Pro	Ser	Leu	Gly
					165					170					175	
	Ala	Gly	Gly	Leu	Gln	Gly	Leu	Ser	Gly	Ala	Gly	Ala	Phe	Asn	Gln	Leu
				180					185					190		
10	Gly	Asn	Ala	Ile	Gly	Met	Gly	Val	Gly	Gln	Asn	Ala	Ala	Leu	Ser	Ala
			195					200					205			
	Leu	Ser	Asn	Val	Ser	Thr	His	Val	Asp	Gly	Asn	Asn	Arg	His	Phe	Val
	210					215					220					
	Asp	Lys	Glu	Asp	Arg	Gly	Met	Ala	Lys	Glu	Ile	Gly	Gln	Phe	Met	Asp
	225					230					235					240
15	Gln	Tyr	Pro	Glu	Ile	Phe	Gly	Lys	Pro	Glu	Tyr	Gln	Lys	Asp	Gly	Trp
					245					250					255	
	Ser	Ser	Pro	Lys	Thr	Asp	Asp	Lys	Ser	Trp	Ala	Lys	Ala	Leu	Ser	Lys
				260					265					270		
20	Pro	Asp	Asp	Asp	Gly	Met	Thr	Gly	Ala	Ser	Met	Asp	Lys	Phe	Arg	Gln
			275					280					285			
	Ala	Met	Gly	Met	Ile	Lys	Ser	Ala	Val	Ala	Gly	Asp	Thr	Gly	Asn	Thr
		290					295						300			
	Asn	Leu	Asn	Leu	Arg	Gly	Ala	Gly	Gly	Ala	Ser	Leu	Gly	Ile	Asp	Ala
	305					310					315					320
25	Ala	Val	Val	Gly	Asp	Lys	Ile	Ala	Asn	Met	Ser	Leu	Gly	Lys	Leu	Ala
					325					330					335	
	Asn	Ala														

30 This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains substantially no cysteine. The *Erwinia chrysanthemi* hypersensitive response elicitor polypeptide or protein is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 22 as follows:

35

CGATTTTACC	CGGGTGAACG	TGCTATGACC	GACAGCATCA	CGGTATTCGA	CACCGTTACG	60
GCGTTTATGG	CCGCGATGAA	CCGGCATCAG	GCGGCGCGCT	GGTCGCCGCA	ATCCGGCGTC	120

- 9 -

	GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACCTCA TGATGCAGAT TCAGCCGGGG	180
	CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG	240
	TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG	300
	CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GGCGGGAATG	360
5	ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC	420
	CGATCATTAA GATAAAGGCG GCTTTTTTTA TTGCAAAACG GTAACGGTGA GGAACCGTTT	480
	CACCGTCGGC GTCACCTCAGT AACAAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG	540
	GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA	600
	AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GGCTGGGTGC	660
10	TCAGGGACTG-AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCAGCG TGGATAAACT	720
	GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT	780
	GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC	840
	TTTCGGCAAT GCGCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA	900
	TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC	960
15	CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC	1020
	CCAGGGTAAT ATGAATGCGT TCGGCAGCGG TGTGAACAAC GCACTGTCGT CCATTCTCGG	1080
	CAACGGTCTC GGCCAGTCGA TGAGTGGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT	1140
	GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT	1200
	GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCAGTAG ACGGTAACAA	1260
20	CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA	1320
	TCAGTATCCG GAAATATTCG GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCGAA	1380
	GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG	1440
	CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA	1500
	TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC	1560
25	GGCTGTCGTC GCGGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA	1620
	ATCTGTGCTG GCCTGATAAA GCGGAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC	1680
	TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA	1740
	ACGCACATTT TCCCGTTCAT TCGCGTCGTT ACGCGCCACA ATCGCGATGG CATCTTCCTC	1800
	GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC	1860

- 10 -

CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTCTATCC GCCCCTTTAG 1920
 CAGATAGATT GCGGTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG 1980
 GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC 2040
 AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG 2100
 5 GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T 2141

The hypersensitive response elicitor polypeptide or protein derived from
Erwinia amylovora has an amino acid sequence corresponding to SEQ. ID. No. 23 as
 10 follows:

	Met	Ser	Leu	Asn	Thr	Ser	Gly	Leu	Gly	Ala	Ser	Thr	Met	Gln	Ile	Ser	
	1				5					10					15		
15	Ile	Gly	Gly	Ala	Gly	Gly	Asn	Asn	Gly	Leu	Leu	Gly	Thr	Ser	Arg	Gln	
				20					25					30			
	Asn	Ala	Gly	Leu	Gly	Gly	Asn	Ser	Ala	Leu	Gly	Leu	Gly	Gly	Gly	Asn	
			35					40					45				
	Gln	Asn	Asp	Thr	Val	Asn	Gln	Leu	Ala	Gly	Leu	Leu	Thr	Gly	Met	Met	
			50				55					60					
20	Met	Met	Met	Ser	Met	Met	Gly	Gly	Gly	Gly	Leu	Met	Gly	Gly	Gly	Leu	
	65					70					75					80	
	Gly	Gly	Gly	Leu	Gly	Asn	Gly	Leu	Gly	Gly	Ser	Gly	Gly	Leu	Gly	Glu	
				85					90					95			
25	Gly	Leu	Ser	Asn	Ala	Leu	Asn	Asp	Met	Leu	Gly	Gly	Ser	Leu	Asn	Thr	
				100					105					110			
	Leu	Gly	Ser	Lys	Gly	Gly	Asn	Asn	Thr	Thr	Ser	Thr	Thr	Asn	Ser	Pro	
				115				120					125				
	Leu	Asp	Gln	Ala	Leu	Gly	Ile	Asn	Ser	Thr	Ser	Gln	Asn	Asp	Asp	Ser	
			130				135					140					
30	Thr	Ser	Gly	Thr	Asp	Ser	Thr	Ser	Asp	Ser	Ser	Asp	Pro	Met	Gln	Gln	
	145					150					155					160	
	Leu	Leu	Lys	Met	Phe	Ser	Glu	Ile	Met	Gln	Ser	Leu	Phe	Gly	Asp	Gly	
				165					170					175			
35	Gln	Asp	Gly	Thr	Gln	Gly	Ser	Ser	Ser	Gly	Gly	Lys	Gln	Pro	Thr	Glu	
				180					185				190				
	Gly	Glu	Gln	Asn	Ala	Tyr	Lys	Lys	Gly	Val	Thr	Asp	Ala	Leu	Ser	Gly	
			195					200					205				

- 11 -

	Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly	
	210	215 220
	Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu	
	225	230 235 240
5	Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln	
		245 250 255
	Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln	
		260 265 270
10	Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe	
		275 280 285
	Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met	
		290 295 300
	Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro	
		305 310 315 320
15	Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser	
		325 330 335
	Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn	
		340 345 350
20	Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn	
		355 360 365
	Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp	
		370 375 380
	Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu	
		385 390 395 400
25	Gly Ala Ala	

This hypersensitive response elicitor polypeptide or protein has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor polypeptide or protein has substantially

30 no cysteine. The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* is more fully described in Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S.-Y. He, A. Collmer, and S. V. Beer, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference. The DNA

- 12 -

molecule encoding this polypeptide or protein has a nucleotide sequence corresponding to SEQ. ID. No. 24 as follows:

	AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA	60
5	GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT	120
	ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG	180
	GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG	240
	GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG	300
	GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GCCTTGGGTG GCTCAGGTGG CCTGGGCGAA	360
10	GGACTGTCTGA ACGCGCTGAA CGATATGTTA GCGCGTTCGC TGAACACGCT GGGCTCGAAA	420
	GGCGGCAACA ATACCACTTC AACAAACAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC	480
	TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC	540
	CCGATGCAGC AGCTGCTGAA GATGTTGAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG	600
	CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC	660
15	GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG	720
	CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC	780
	GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG	840
	TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT	900
	ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG	960
20	GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGCAA GCCGCAGTAC	1020
	CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAA AGCACTGAGC	1080
	AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC	1140
	ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC	1200
	GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA	1260
25	CTTGGCAAGC TGGGCGCGGC TTAAGCTT	1288

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 25

as follows:

- 13 -

	Met	Gln	Ser	Leu	Ser	Leu	Asn	Ser	Ser	Ser	Leu	Gln	Thr	Pro	Ala	Met	
	1				5					10					15		
	Ala	Leu	Val	Leu	Val	Arg	Pro	Glu	Ala	Glu	Thr	Thr	Gly	Ser	Thr	Ser	
				20					25					30			
5	Ser	Lys	Ala	Leu	Gln	Glu	Val	Val	Val	Lys	Leu	Ala	Glu	Glu	Leu	Met	
			35					40					45				
	Arg	Asn	Gly	Gln	Leu	Asp	Asp	Ser	Ser	Pro	Leu	Gly	Lys	Leu	Leu	Ala	
		50					55					60					
10	Lys	Ser	Met	Ala	Ala	Asp	Gly	Lys	Ala	Gly	Gly	Gly	Ile	Glu	Asp	Val	
	65					70					75					80	
	Ile	Ala	Ala	Leu	Asp	Lys	Leu	Ile	His	Glu	Lys	Leu	Gly	Asp	Asn	Phe	
					85					90					95		
	Gly	Ala	Ser	Ala	Asp	Ser	Ala	Ser	Gly	Thr	Gly	Gln	Gln	Asp	Leu	Met	
				100					105					110			
15	Thr	Gln	Val	Leu	Asn	Gly	Leu	Ala	Lys	Ser	Met	Leu	Asp	Asp	Leu	Leu	
				115				120					125				
	Thr	Lys	Gln	Asp	Gly	Gly	Thr	Ser	Phe	Ser	Glu	Asp	Asp	Met	Pro	Met	
		130					135					140					
20	Leu	Asn	Lys	Ile	Ala	Gln	Phe	Met	Asp	Asp	Asn	Pro	Ala	Gln	Phe	Pro	
	145					150					155					160	
	Lys	Pro	Asp	Ser	Gly	Ser	Trp	Val	Asn	Glu	Leu	Lys	Glu	Asp	Asn	Phe	
					165					170					175		
	Leu	Asp	Gly	Asp	Glu	Thr	Ala	Ala	Phe	Arg	Ser	Ala	Leu	Asp	Ile	Ile	
				180					185					190			
25	Gly	Gln	Gln	Leu	Gly	Asn	Gln	Gln	Ser	Asp	Ala	Gly	Ser	Leu	Ala	Gly	
			195					200					205				
	Thr	Gly	Gly	Gly	Leu	Gly	Thr	Pro	Ser	Ser	Phe	Ser	Asn	Asn	Ser	Ser	
		210					215					220					
30	Val	Met	Gly	Asp	Pro	Leu	Ile	Asp	Ala	Asn	Thr	Gly	Pro	Gly	Asp	Ser	
	225					230					235					240	
	Gly	Asn	Thr	Arg	Gly	Glu	Ala	Gly	Gln	Leu	Ile	Gly	Glu	Leu	Ile	Asp	
					245					250					255		
	Arg	Gly	Leu	Gln	Ser	Val	Leu	Ala	Gly	Gly	Gly	Leu	Gly	Thr	Pro	Val	
				260					265					270			
35	Asn	Thr	Pro	Gln	Thr	Gly	Thr	Ser	Ala	Asn	Gly	Gly	Gln	Ser	Ala	Gln	
			275					280					285				

- 14 -

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
 290 295 300
 Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
 305 310 315 320
 5 Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
 325 330 335
 Asn Gln Ala Ala Ala
 340

10

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine.

Further information about the hypersensitive response elicitor derived from
Pseudomonas syringae is found in He, S. Y., H. C. Huang, and A. Collmer,
 15 "*Pseudomonas syringae* pv. *syringae* Harpin_{PS}: a Protein that is Secreted via the Hrp
 Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255-1266
 (1993), which is hereby incorporated by reference. The DNA molecule encoding the
 hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide
 sequence corresponding to SEQ. ID. No. 26 as follows:

20

ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCCTG 60
 GTACGTCTCG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC 120
 GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA 180
 AAACGTGTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC 240
 25 ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG 300
 GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC 360
 AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC 420
 GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC 480
 AAGCCGGA CTGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC 540
 30 GAAACGGCTG CGTTCCGTTC GGCACTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG 600
 AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTC 660
 AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC 720
 GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA 780
 TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCAGAC CCGTACGTCG 840

- 15 -

GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG 900
 GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT 960
 GCGCAAATCG CCACCTTGCT GGTCACTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA 1020
 GCCTGA 1026

5

The hypersensitive response elicitor polypeptide or protein derived from
Pseudomonas solanacearum has an amino acid sequence corresponding to SEQ. ID.
 No. 27 as follows:

10	Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln	1	5	10	15
	Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser	20	25	30	
15	Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile	35	40	45	
	Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly	50	55	60	
	Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala	65	70	75	80
20	Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser	85	90	95	
	Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met	100	105	110	
25	Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala	115	120	125	
	Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val	130	135	140	
	Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala	145	150	155	160
30	Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly	165	170	175	
	Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly	180	185	190	
35	Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala	195	200	205	

- 16 -

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
 210 215 220
 Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
 225 230 235 240
 5 Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
 245 250 255
 Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
 260 265 270
 10 Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
 275 280 285
 Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
 290 295 300
 Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
 305 310 315 320
 15 Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
 325 330 335
 Gln Ser Thr Ser Thr Gln Pro Met
 340

It is encoded by a DNA molecule having a nucleotide sequence corresponding SEQ.

20 ID. No. 28 as follows:

ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC 60
 AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC 120
 GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC 180
 GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC 240
 25 AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC 300
 GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA 360
 GACCTGGTGA AGCTGCTGAA GCGGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG 420
 GGCAACGGCG TGGGCGGTGC CAACGCGGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC 480
 GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC 540
 30 GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT 600
 GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC 660
 GGCCCCGAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC 720
 CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG 780

- 17 -

```

ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC      840
GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT      900
GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCAGATCA TGGATGTGGT GAAGGAGGTC      960
GTCCAGATCC TGCAGCAGAT GCTGGCGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG     1020
5  ACGCAGCCGA TGTAAT                                     1035

```

Further information regarding the hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* is set forth in Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pemollet, and C. A. Boucher, "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference.

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris* pv. *glycines* has an amino acid sequence corresponding to SEQ. ID. No. 29 as follows:

```

      Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Ala Ile Leu Ala
      1              5              10              15
20  Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr
      20              25

```

This sequence is an amino terminal sequence having only 26 residues from the hypersensitive response elicitor polypeptide or protein of *Xanthomonas campestris* pv. *glycines*. It matches with fimbrial subunit proteins determined in other *Xanthomonas campestris* pathovars.

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris* pv. *pelargonii* is heat stable, protease sensitive, and has a molecular weight of 20 kDa. It includes an amino acid sequence corresponding to SEQ. ID. No. 30 as follows:

```

      Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
      1              5              10              15
35  Leu Leu Ala Met
      20

```

Isolation of *Erwinia carotovora* hypersensitive response elicitor protein or polypeptide is described in Cui et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp* N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is
5 hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact., July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am.
10 Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference.

Hypersensitive response elicitor proteins or polypeptides from *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamoni*, *Phytophthora capsici*, *Phytophthora megasperma*, and *Phytophthora citrophthora* are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and
15 Induction of Resistance to Bacterial and Fungal Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-25 (1993), Ricci et al., "Structure and Activity of Proteins from Pathogenic Fungi Phytophthora Eliciting Necrosis and Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989), Ricci et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of
20 *Phytophthora parasitica*," Plant Path., 41:298-307 (1992), Baillreul et al., "A New Elicitor of the Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defence Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J.
25 Plant Path., 102:181-92 (1996), which are hereby incorporated by reference.

The above elicitors are exemplary. Other elicitors can be identified by growing fungi or bacteria that elicit a hypersensitive response under which genes encoding an elicitor are expressed. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e. local necrosis) by using them to infiltrate
30 appropriate plant tissues.

Fragments of the above hypersensitive response elicitor polypeptides or proteins as well as fragments of full length elicitors from other pathogens are encompassed by the method of the present invention.

Suitable fragments can be produced by several means. In the first, subclones
5 of the gene encoding a known elicitor protein are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by
10 digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

15 In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for expression of a truncated peptide or protein.

20 Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

25 An example of suitable fragments of an *Erwinia* hypersensitive response elicitor which fragments elicit a hypersensitive response are fragments of the *Erwinia amylovora* hypersensitive response elicitor. Suitable fragments include a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, or an internal fragment of the amino
30 acid sequence of SEQ. ID. No. 23. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 can span amino acids 105 and 403 of SEQ. ID. No. 23. The N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 can span the

- 20 -

following amino acids of SEQ. ID. No. 23: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The internal fragment of the amino acid sequence of SEQ. ID. No. 23 can span the following amino acids of SEQ. ID. No. 23: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180. Other suitable fragments can be identified in accordance with the present invention.

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

The fragment of the present invention is preferably produced in purified form (preferably at least about 60%, more preferably 80%, pure) by conventional techniques. Typically, the fragment of the present invention is produced but not secreted into the growth medium of recombinant host cells. Alternatively, the protein or polypeptide of the present invention is secreted into growth medium. In the case of unsecreted protein, to isolate the protein fragment, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to heat treatment and the fragment is separated by centrifugation. The supernatant fraction containing the fragment is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the fragment. If necessary, the protein fraction may be further purified by ion exchange or HPLC.

The DNA molecule encoding the fragment of the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector

- 21 -

contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression

- 22 -

elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

5 Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic
10 signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient
15 translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably
20 promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription).
25 For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promotors such as the T7 phage promotor, *lac* promotor, *trp* promotor, *recA*
30 promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a

- 23 -

hybrid *trp-lacUV5 (tac)* promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

5 Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

10 Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or
15 translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or
20 from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the fragment of a hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it
25 is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention further relates to methods of imparting disease
30 resistance to plants, enhancing plant growth, and/or effecting insect control for plants. These methods involve applying the fragment of a hypersensitive response elicitor polypeptide or protein, which fragment itself elicits a hypersensitive response, in a

- 24 -

non-infectious form to all or part of a plant or a plant seed under conditions effective for the fragment to impart disease resistance, enhance growth, and/or control insects. Alternatively, these fragments of a hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to enhance plant growth, and/or to effect insect control.

As an alternative to applying a fragment of a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, and/or to control insects on the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a fragment of a hypersensitive response elicitor polypeptide or protein, which fragment elicits a hypersensitive response, and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a fragment of a hypersensitive response elicitor polypeptide or protein which fragment elicits a hypersensitive response can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, and/or to control insects.

The embodiment of the present invention where the hypersensitive response elicitor polypeptide or protein is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of an isolated fragment or 2) application of bacteria which do not cause disease and are transformed with a genes encoding the fragment. In the latter embodiment, the fragment can be applied to plants or plant seeds by applying bacteria containing the DNA molecule encoding the fragment of the hypersensitive response elicitor polypeptide or protein which fragment elicits a hypersensitive response. Such bacteria must be capable of secreting or exporting the fragment so that the fragment can contact plant or plant seeds cells. In these embodiments, the fragment is produced by the bacteria *in planta* or on seeds or just prior to introduction of the bacteria to the plants or plant seeds.

- 25 -

The methods of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, and/or control insects. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the fragments of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases, treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solanacearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthomonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the fragments of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased

- 26 -

quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops
5 to be grown in areas where short growing seasons would otherwise preclude their growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of
10 insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants,
15 preventing insects from colonizing host plants, preventing colonizing insects from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on over 200
20 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm),
25 pepper maggot, tomato pinworm, and maggots. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

The method of the present invention involving application of the fragment of a hypersensitive response elicitor polypeptide or protein, which fragment elicits a
30 hypersensitive response, can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein

- 27 -

into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds or propagules (e.g., cuttings), in accordance with the application embodiment of the present invention, the fragment of the hypersensitive response elicitor protein or polypeptide, in accordance with present invention, can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the fragment with cells of the plant or plant seed. Once treated with the fragment of the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the fragment of the hypersensitive response elicitor protein or polypeptide or whole elicitors to impart disease resistance to plants, to enhance plant growth, and/or to control insects on the plants.

The fragment of the hypersensitive response elicitor polypeptide or protein, in accordance with the present invention, can be applied to plants or plant seeds alone or in a mixture with other materials. Alternatively, the fragment can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a fragment of a hypersensitive response elicitor polypeptide or protein which fragment elicits a hypersensitive response in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM of the fragment.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematocide, and mixtures thereof. Suitable fertilizers include $(\text{NH}_4)_2\text{NO}_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response eliciting fragment can

- 28 -

be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a hypersensitive response eliciting fragment
5 need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding such a fragment are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The
10 genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic
15 transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions
20 effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g.,
25 dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by
30 reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is

- 29 -

hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

- 30 -

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response eliciting fragment resulting in disease resistance, enhanced plant growth, and/or control of insects on the plant. Alternatively, transgenic seeds or propagules (e.g., cuttings) are recovered from the transgenic plants. The seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, and/or to control insects. While not wishing to be bound by theory, such disease resistance, growth enhancement, and/or insect control may be RNA mediated or may result from expression of the polypeptide or protein fragment.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response eliciting fragment is applied. These other materials, including hypersensitive response eliciting fragments,

- 31 -

- can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response eliciting
- 5 fragment to impart disease resistance, enhance growth, and/or control insects. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

EXAMPLES

10

Example 1 - Strains and plasmids used

The strains and plasmids used are set forth in Table 1 below

15

Table 1

Plasmid name	<i>E. amylovora</i> source strain	Brief Description, Relevant Phenotype, Reference	Harpin, fragment (or NA)
pBCKS	-	Cm ^r derivative of pBluescript KS. Stratagene, La Jolla, CA	
pBCSK	-	Cm ^r derivative of pBluescript SK. Stratagene, La Jolla, CA	
pBSKS	-	pBluescript KS; Ap ^r . Stratagene, La Jolla, CA	
pBSSK II	-	pBluescript II SK; Ap ^r . Stratagene, La Jolla, CA	
pBW7	-	Mob+Tc ^r helper plasmid. (Rella, et al., "Transposon Insertion Mutagenesis of <i>Pseudomonas aeruginosa</i> With a Tn5 Derivative: Application to Physical Mapping of the <i>arc</i> Gene Cluster," <i>Gene</i> 33:293-303 (1985), which is hereby incorporated by reference)	NA
pCPP51	-	Broad host range derivative of pBSSK II containing <i>ori</i> from pRO1614.	NA
PCPP430	321	Functional <i>hrp</i> gene cluster of <i>E. amylovora</i> Ea321 cloned into pCPP9. Beer, S.V., et al., "The <i>hrp</i> Gene Cluster of <i>Erwinia Amylovora</i> ," In Hennecke, H., and D.P.S. Verma, (eds.), <i>Advances in Molecular Genetics of Plant-Microbe Interactions</i> , Kluwer Academic Publishers, Dordrecht, Netherlands, 53-60 (1991), which is hereby incorporated by reference.	
pCPP460	246	Functional <i>hrp</i> gene cluster of <i>E. amylovora</i> Ea246 cloned into pCPP9.	
pCPP1104	321	1.2 kb <i>Pst</i> I fragment of pCPP1084 in pBCKS	Ea C31
pCPP1105	321	1.1 kb <i>Sma</i> I fragment of pCPP1084 in pBCSK	Ea C82
pCPp1107	321	1.3 kb <i>Hind</i> III fragment of pCPP1084 in pBCSK	Ea wt
pCPp1108	321	1.2 kb <i>Hinc</i> II- <i>Hind</i> III of pCPP1084 in pBCSK	Ea N11
pCPP1109	321	pCPP1107 with internal <i>Ava</i> II fragment deleted	Ea 1175
pCPP1110	321	As pCPP1108, but cloned into pBCKS	Ea N9
pCPP1111	321	367 bp <i>Taq</i> I fragment of pCPP1107 in pBCSK	Ea C305

pCPP1113	246	As pCPP1109, but 425bp <i>Avall</i> fragment of pCPP1098 deleted	Ear 1175
pCPP1119	246	Site specific mutation in pCPP1098; stop codon inserted at L36	Ear C368
pCPP1120	246	Site specific mutation in pCPP1098; stop codon inserted at T123	Ear C281
pCPP1121	321	702bp <i>KpnI</i> fragment internal to <i>hrpN</i> deleted	Ea C375
pCPP1127	246	3.1 kb <i>BamHI</i> fragment of pCPP1098 in pSU21	Ear wt
pCPP1128	246	Tn10 minikan in pCPP1127	Ear undef
pCPP1136	246	4.4 kb <i>EcoRI</i> fragment of pCPP1120, religated	EAR N122
pCPP1146	246	4.2 kb <i>EcoRI</i> fragment of pCPP1119, religated	Ear N35
pCPP1147	321	1.2 kb <i>BamHI</i> fragment of pCPP1084, PCR amplified, cloned into pSU23	Ea wt
pCPP1148	246	As pCpP1147, but from pCPP1098	Ear wt
pCPP1150	246	As pCpP1148, but in pCPP51 vector	Ear wt
pCPP1163	246	3.1 kb <i>BamHI</i> fragment of pCPP1098 in pCPP51	Ear wt
pCPP1164	321	1.3 kb <i>HindIII</i> of pCpP1084 in pCPP51	Ea wt
pCPP1165		Derivative of pCPP51 w / <i>KpnI</i> , <i>SacII</i> sites removed	NA
pCPP1167	321	1.3 kb <i>HindII</i> fragment of pCpP1107 in pCPP1165	Ea wt
pCPP1169	246	As pCPP1167, but 3.1 kb <i>BamHI</i> insert from pCpP1098	Ear wt
pCPP1170	246	PCPP1098; Σ -Sp ligated into <i>EcoRV</i> site	Ear C128 Σ
pCPP1171	246	<i>KpnI</i> fragment internal to <i>hrpN</i> deleted; shifted reading frame	Ea C375
pCPP1172	321	Derivative of pCPP1167 with in-frame deletion of <i>KpnI</i> fragment internal to <i>hrpN</i>	Ea 1235
pCPP1173	246	As pCPP1172, but from pCPP1169	Ear 1235
pCPP217	321	PCPP1084 with 2 <i>StyI</i> fragments deleted, blunted, and religated	Ea C185
pCPP1252	321	PCPP1105 with Σ -Sp ligated at <i>SmaI</i> site	Ea C82 Σ
pGP1-2		P15a ori.T7 RNA polymerase-encoding plasmid; for protein expression (Tabor, S., et al., "A Bacteriophage T7 DNA Polymerase/Promoter System for Controlled Exclusive Expression of Specific Genes," <i>Proc. Natl. Acad. Sci. USA</i> , 82:1074-1078 (1985), which is hereby incorporated by reference).	N/A
pHP45 Σ Ω		Ap ^r ; Sp ^r ; source of Ω -Sp fragment; (Fellay, R., et al., "Interposon Mutagenesis of Soil and Water Bacteria a Family of DNA Fragments Designed for <i>in vitro</i> Insertional Mutagenesis of Gram-Negative Bacteria," <i>Gene</i> , 52:147-154 (1987), which is hereby incorporated by reference).	N/A
pSU21		P15a ori Km ^r (Bartolomé, B.Y., et al., "Construction and properties of a Family of pACYC184-Derived Cloning Vectors Compatible With pBR322 and its Derivatives," <i>Gene</i> , 102:75-78 (1991), which is hereby incorporated by reference).	N/A
PSU23		P15a ori Km ^r (Bartolomé, B.Y., et al., "Construction and properties of a Family of pACYC184-Derived Cloning Vectors Compatible With pBR322 and its Derivatives," <i>Gene</i> , 102:75-78 (1991), which is hereby incorporated by reference).	N/A
Strains used			
<i>E. amylovora</i>		Ea273Nx; Nalidixic acid resistant (Nx ^r) derivative of Ea273. CUCPB 2348	
<i>E. amylovora</i>		Rifampin resistant derivative of Ea32. CUCPB 2545	

<i>E. coli</i>	GM272; <i>dam</i> -, <i>dcm</i> -. CUCPB 3047; (Blumenthal, R.M., et al., "E. coli Can Restrict Methylated DNA and May Skew Genomic Libraries," <u>Trends in Biotech.</u> , 4:302-305 (1986), which is hereby incorporated by reference)
<i>E. coli</i>	BL21(DE3); CUCPB 4277; (Studier, F.W., and B.A. Moffatt, "Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-level Expression of Cloned Genes," <u>J. Mol. Biol.</u> , 189:113-130 (1986), which is hereby incorporated by reference)
<i>E. coli</i>	DH5 α ; (Nx ^r). CUCPB 2475; Stratagene, La Jolla, CA.

Example 2 - Molecular biology techniques.

- 5 Several approaches were employed to obtain truncated or otherwise altered versions of both *E. amylovora* harpins. These techniques included: (i) subcloning of restriction fragments containing portions of the gene encoding the hypersensitive response elicitor protein or polypeptide from *Erwinia amylovora* (i.e. *hrpN*) into expression vectors, by standard techniques (Sambrook, et al., Molecular Cloning: a
- 10 Laboratory Manual, 2nd ed. ed. Cold Spring Harbor, Laboratory," Cold Spring Harbor, NY (1989), which is hereby incorporated by reference); (ii) insertion of an Ω -fragment (Fellay, et al., "Interposon Mutagenesis of Soil and Water Bacteria a Family of DNA Fragments Designed for *in vitro* Insertional Mutagenesis of Gram-Negative Bacteria," Gene 52:147-154 (1987), which is hereby incorporated by reference) into
- 15 *hrpN*; (iii) site-specific mutagenesis approaches (Innis, et al., PCR Protocols. A Guide to Methods and Applications, Academic Press San Diego, CA (1990); Kunkel, et al., "Rapid and Efficient Site-Specific Mutagenesis Without Phenotypic Selection," Proc. Nat. Acad. Sci. USA 82:488-492 (1985), which are hereby incorporated by reference); and (iv) creation of nested deletions (Erase-a-BaseTM kit; Promega,
- 20 Madison, WI). C-terminal deletion analysis of the hypersensitive response elicitor protein or polypeptide from *Erwinia amylovora* (i.e. harpin_{Ea}) in pCPP1084 could not be performed because of the location of restriction enzyme cleavage sites in pCPP1084. For N-terminal deletions, pCPP1084 DNA was prepared using a Qiagen midiprep column (Qiagen, Chatsworth, CA) and digested with *sst I* followed by
- 25 *EcoRI*. Subsequently, the digested DNA was subjected to exonuclease III digestion, ligation, and transformation into *E. coli* BL21(DE3). Deletion sizes were estimated by agarose gel electrophoresis. Harpin fragments were named with respect to the portion of harpin deleted (e.g., harpin_{Ea} C82 lacks the C-terminal 82 amino acid residues of full-length harpin_{Ea}).

Example 3 - Prot in expression.

For expression from T7 promoters, T7 RNA polymerase-dependent systems were used. These systems utilized either strain *E. coli* BL21(DE3) (Studier, et al.,
5 "Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Gene," J. Mol. Biol. 189:113-130 (1986), which is hereby incorporated by reference), or plasmid pGP1-2 (Tabor, et al., "A Bacteriophage T7 DNA Polymerase/Promoter System for Controlled Exclusive Expression of Specific Genes," Proc. Natl. Acad. Sci., USA 82:1074-1078 (1985), which is hereby
10 incorporated by reference) in *E. coli* DH5 α . Expression of *hrpN* from the T7 promoter was induced by addition of IPTG to a final concentration of 0.4 mM. For expression in *E. amylovora* Ea321 (i.e. harpin_{Ea}) or Ea273, pGP1-2 was introduced by transformation with a 42°C heat shock for 10 minutes, or by electroporation (Biorad Gene PulserTM). Hypersensitive response (i.e. HR)-eliciting activity was screened in
15 tobacco cv. Xanthi leaves by *in planta* lysis (He, et al., "*Pseudomonas syringae* pv. *syringae* harpin_{PSS}: a Protein That is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference) or by preparation of boiled and unboiled "CFEPs" (Wei, et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen
20 *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference).

Example 4 - In vitro proteolysis of harpin.

25 *In vitro* proteolysis of harpin_{Ea} with *Staphylococcus* V8 proteinase (also termed endoproteinase Glu-C), trypsin, pepsin, and papain was performed as recommended (Scopes, et al., Protein Purification: Principles and Practice, 2nd ed. Springer-Verlag. New York (1987), which is hereby incorporated by reference), for 2-16 hrs. at 20-37°. Endoproteinase Glu-C digestion was performed either in 50 mM
30 ammonium bicarbonate, pH 7.8 (in which cleavage occurs only after glutamic acid), or in 50 mM potassium phosphate, pH 7.8 (in which cleavage after both glutamic acid and aspartic acid occurs).

Example 5 - Plant-derived proteinases.

Intercellular fluids (IF) were obtained from tobacco, tomato, apple, raspberry, and cotoneaster, as described (Hammond-Kosack, et al., "Preparation and Analysis of
5 Intercellular Fluid," p. 15-21. *In* S.J. Gurr, M.J. McPherson, and D.J. Bowles (ed.), Molecular Plant Pathology A Practical Approach, 2nd ed., The Practical Approach Series, IRL Publishers, Oxford (1992), which is hereby incorporated by reference), by vacuum infiltration of intercellular spaces with high-purity water. Proteolytic digestion of PAGE-purified harpin_{Ea} was performed for 2-16 hrs. at 20-37°C, pH, by
10 mixing equal volumes of IF with harpin_{Ea}. A total leaf extract was obtained by grinding tobacco leaf panels with mortar and pestle in 5 mM potassium phosphate. The extract was centrifuged and filtered, and the clarified ground leaf extract used identically as was the IF. Proteinase inhibitors were employed as follows: Pepstatin A (final concentration 1µM), E-64 (1µM), Aprotinin (2µg/ml), o-phenanthroline
15 (1mM), and p-mercuribenzoate (PCMB) (Sigma, St. Louis, MO).

Example 6 - Peptide purification.

Peptide fragments of harpin obtained following digestion with tobacco IF were
20 fractionated by reverse-phase HPLC on a Vydac C18 column using a 2-60% acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions were lyophilized, resuspended in 5 mM potassium phosphate and infiltrated into tobacco leaf panels. The fraction with greatest HR-eliciting activity was refractionated as above with a 35-70% acetonitrile gradient, and the purity of each fraction was assayed via gas
25 chromatography-mass spectroscopy (GC-MS) and by N-terminal protein sequencing at the Cornell Biotechnology Program Core Facility.

Example 7 - Proteinase activity-stained gels.

30 Proteinase activity of IF was assayed in activity-stained polyacrylamide gels (Laemmli, "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4," Nature 227:680-685 (1970), which is hereby incorporated by reference) copolymerized with 0.1% gelatin (Heussen, et al., "Electrophoretic Analysis of Plasminogen Activators in Polyacrylamide Gels Containing Sodium

- 36 -

Dodecyl Sulfate and Copolymerized Substrates," Anal. Biochem. 102:196-202 (1980), which is hereby incorporated by reference). After electrophoresis, each gel was rinsed extensively to remove SDS and allow refolding of proteinases in the gel. Following additional incubation to allow proteolysis to occur, the gels were stained
5 with 0.1% Amido Black in 30% methanol/10% acetic acid. Each gel stained darkly (due to the presence of copolymerized gelatin) except where proteinases had digested the gelatin, resulting in colorless bands representing the sites of proteinase activity.

10 **Example 8 - Truncated harpins retain HR-eliciting activity.**

The stability and the HR-eliciting activity of proteins encoded by various DNA constructs is shown in Figure 1. Many DNA constructs encoding portions of harpin_{Ea} or harpin_{Ea}r did not yield detectable protein products following induction of expression in the T7 promoter-polymerase system (Tabor, et al., "A Bacteriophage T7
15 DNA Polymerase/Promoter System for Controlled Exclusive Expression of Specific Genes," Proc. Natl. Acad. Sci. USA 82:1074-1078 (1985), which is hereby incorporated by reference) and analysis of cell extracts by PAGE, possibly due to instability of the encoded proteins. No DNA constructs (e.g., those obtained via Erase-a-BaseTM protocol) yielded detectable protein products displaying N-terminal
20 deletions relative to the full-length protein. No stable but inactive proteins were identified. Several constructs encoding proteins truncated at their C-terminus and often including additional vector-encoded amino acids yielded detectable products (e.g. harpin_{Ea} C82). In contrast, a construct encoding the same 321 N-terminal amino acid residues of harpin_{Ea}, but yielding a protein truncated by the presence of an Ω-
25 fragment (harpin_{Ea} C82 Ω) was unstable (i.e. no product was detected). A construct encoding a harpin_{Ea} fragment with a large internal deletion (harpin_{Ea} I175) was also successfully used to express protein. These various truncated proteins were tested for HR-eliciting activity. A 98 residue N-terminal harpin_{Ea} fragment (harpin_{Ea} C305) was the smallest bacterially-produced peptide that displayed HR-eliciting activity.

30

- 37 -

Example 9 - Secretion of harpin_{Ea} with an altered C-terminus.

The effect of alteration at the harpin C-terminus on its secretion was examined. Harpin C31 contains the N-terminal 372 amino acids of harpin, but lacks
5 the C-terminal 31 residues, which are replaced by 47 residues encoded by the vector, resulting in a protein slightly larger than the wild type harpin_{Ea}. The C31 protein retains HR-eliciting activity and is stable and easily expressed and detected by western analysis or PAGE but it is no longer secreted into the culture supernatant as is the wild type protein (Figure 2). The presence of harpin_{Ea} C31 does not interfere with
10 secretion of the wild type harpin, which is found in both the CFEP and the culture supernatant. However, harpin_{Ea} C31 is found only in the CFEP.

Example 10 - Effect of proteolysis on harpin_{Ea}'s HR eliciting activity

15 In order to generate additional harpin_{Ea} fragments, purified full length protein was proteolyzed *in vitro* by several proteinases, including endoproteinase Glu-C, trypsin, pepsin, and papain (e.g., Figures 3 and 4). Harpin solutions digested with trypsin or with papain lost all activity. In contrast, following digestion with endoproteinase Glu-C, HR-eliciting activity was retained. No peptides larger than 6
20 kD were evident by PAGE following trypsin digestion. Endoproteinase Glu-C digestion yielded an approximately 20 kD fragment, larger than expected if all cleavage sites were cut, indicating that digestion was not complete (Figure 4).

Example 11 - Apoplastic fluids (IF) contain harpin-degrading proteolytic activity

25 Apoplastic fluids (intercellular fluids; IF) from tobacco and other plants were also employed to proteolyze harpin. Each IF tested possessed proteinase activity(s), as indicated by the presence of multiple activity-stained bands in polyacrylamide gels containing co-polymerized gelatin (Figures 5A to 5C), as well as by the disappearance of detectable harpin_{Ea} (Schägger, et al., "Tricine-Sodium Dodecyl Sulfate Gel
30 Electrophoresis for the Separation of Proteins in the Range From 1 to 100 kDa," Anal. Biochem. 166:368-379 (1987), which is hereby incorporated by reference) following overnight digestion of purified harpin_{Ea} with IF. Proteinase activity was substantially greater at 37°C than at 20°C, and activity was higher at pH 8.5 than at pH 7. Several

- 38 -

inhibitors were employed in order to define the proteolytic activity(s) of the IF. No single proteinase inhibitor which was employed prevented degradation of harpin_{Ea}. However, a mixture of the inhibitors Pepstatin A (1μM), E-64 (1μM), Aprotinin (2μg/ml), and o-phenanthroline (1mM), targeted at acid proteinases, cysteine
5 proteinases, serine proteinases, and metalloproteinases, respectively, partially inhibited proteolysis.

Harpin_{Ea} degraded by proteolytic activities present in the plant apoplast retained HR-eliciting activity (Figure 3). In contrast, harpin_{Ea} proteolyzed by a clarified extract produced by grinding tobacco leaf tissue with mortar and pestle lost
10 HR-eliciting activity. In order to study whether apoplastic degradation of harpin was a prerequisite to its HR-eliciting activity, the length of time required for leaf collapse when either intact harpin or harpin predigested with tobacco IF was infiltrated into tobacco leaf panels was compared. Both preparations elicited the HR in a similar time frame (12-18 hours, depending on the experiment).

15

Example 12 - Characterization of HR-eliciting peptide fragments

Peptides resulting from digestion by apoplastic plant proteinase(s) were fractionated by reverse phase HPLC (Vydac C18 column), and tested for activity.
20 Following treatment of intact harpin_{Ea} with tobacco IF, three fractions contained some HR-eliciting activity on tobacco. Two of the three demonstrated weak activity, and little protein was present. They were not further characterized. Fraction 19, which contained the strongest activity as well as the most protein, was refractionated using a more shallow elution gradient (Figure 6). Refractionation, N-terminal protein
25 sequencing, and molecular weight analysis by mass spectroscopy indicated that four largely overlapping peptides were present. Peak 19-1 contained peptides P91 and P95, corresponding to harpin_{Ea} residues 110-200 and 110-204; peak 19-2 contained peptides P64 and P68, corresponding to harpin_{Ea} residues 137-200 and 137-204. 19-1 and 19-2 each possessed HR-eliciting activity. The smallest peptide thus confirmed
30 to retain activity consisted of residues 137-204. The two peptides in each peak were not separable under the conditions used. These active fragments are distinct from the smallest active N-terminal fragment (harpin_{Ea}C305), and indicate that more than one portion of harpin_{Ea} displays activity *in planta*. Further digestion with trypsin

- 39 -

abolished the HR-eliciting activity of 19-2. This proteinase cleaves P64 and P68 as shown in Figure 7. Further digestion with endoproteinase Glu-C in ammonium bicarbonate buffer abolished the HR-eliciting activity of 19-1. Endoproteinase Glu-C is predicted to cleave P91 and P95 as shown in Figure 7. Loss of elicitor-activity followed further digestion of these peptides with endoproteinase Glu-C or trypsin.

Example 13 - *E. amylovora* harpin's similarity with other proteins.

The predicted protein sequences of proteinaceous HR elicitors from several other bacterial plant pathogens, and of other proteins known to be, or thought to be, secreted by a type III secretion pathway were also compared with that of harpin_{Ea}. When harpin_{Ea} was compared with elicitors from *E. amylovora* Ea246 (i.e. harpin_{Ea}), *Erwinia chrysanthemi* EC16 (harpin_{Ech}) (Bauer, et al., "Erwinia chrysanthemi harpin_{Ech}: An Elicitor of the Hypersensitive Response That Contributes to Soft-Rot Pathogenesis," Mol. Plant-Microbe Interact 8:484-491 (1995), which is hereby incorporated by reference), *Erwinia carotovora* subsp. *carotovora* (harpin_{Ecc}) (Mukherjee, et al., Presented at the 8th International Congress Molecular Plant-Microbe Interactions, Knoxville, TN (1996), which is hereby incorporated by reference), *Erwinia stewartii* (Harpin_{Es}) (Frederick, et al., "The *wts* Water-Soaking Genes of *Erwinia stewartii* are Related to *hrp* genes," Presented at the Seventh International Symposium on Molecular Plant-Microbe Interactions, Edinburgh, Scotland (1994), which is hereby incorporated by reference), *Ralstonia* (*Pseudomonas*) *solanacearum* (PopA) (Arlat, et al., "PopA1, a Protein Which Induces a Hypersensitivity-Like Response on Specific *Petunia* Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-553 (1994), which is hereby incorporated by reference), *Pseudomonas syringae* 61 (harpin_{Pss}) (He, et al., "Pseudomonas syringae pv. syringae harpin_{Pss}: a Protein That is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference), *Pseudomonas syringae* pv. *tomato* (harpin_{Pst}) (Preston, et al., "The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* Are Encoded By An Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato But Not Soybean," Mol. Plant-Microbe Interact 8:717-732 (1995), which is hereby incorporated by reference),

- 40 -

the *Erwinia*-derived harpins contained significant regions of similarity at the C-terminus. In addition, all the elicitors are glycine-rich, secreted, and heat-stable. Limited similarity between harpin_{PSS} and harpin_{Ea} had been reported previously (He, et al., "*Pseudomonas syringae* pv. *syringae* harpin_{PSS}: a Protein That is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference), (Laby, et al., Presented at the Seventh International Symposium on Molecular Plant-Microbe Interactions, Edinburgh, Scotland (1994), which is hereby incorporated by reference). A limited region of similarity between harpin_{Ea} and other harpins from *Erwinia* spp. was also evident at the extreme N-terminus of each protein, where 9 out of the first 26 residues are conserved (Figure 8). Kyte-Doolittle hydropathy plots for each of the harpins produced by different *Erwinia* spp. are illustrated in Figure 9. Each of the *Erwinia* harpins examined displays a generally similar hydrophobicity profile along the full length of the protein. This profile is distinct from the profile demonstrated by PopA1 and by harpin_{PSS}, and does not possess the symmetry evident in the profile of those two proteins. The hydropathy profile of each *Erwinia* harpin is generally similar to that of the others, yet distinct from that reported for harpin_{PSS} (Alfano, et al., "Analysis of the Role of the *Pseudomonas Syringae* HrpZ harpin in Elicitation of the Hypersensitive Response to Tobacco Using Functionally Nonpolar *hrpZ* Deletion Mutations, Truncated HrpZ Fragments, and *hrmA* Mutations," Mol. Microbiol. 19:715-728 (1996), which is hereby incorporated by reference). Harpin_{Ecc} possesses a strikingly hydrophobic region around residues 54-143 (Mukherjee, et al., Presented at the 8th International Congress Molecular Plant-Microbe Interactions, Knoxville, TN (1996), which is hereby incorporated by reference). This portion of the protein is also the most hydrophobic region of harpin_{Ea} and harpin_{Es}. The rest of each protein is predominantly hydrophilic.

Truncated proteins and fragments of harpin obtained following proteolytic digestion of the full length protein indicate several surprising aspects of harpin_{Ea} HR-eliciting activity. These harpin fragments demonstrate that HR-eliciting activity resides in distinct regions of the protein, and that relatively small fragments of the protein, as little as 68 residues and possibly less, are sufficient for this activity. Fragments of other plant pathogen-derived elicitor proteins also retain biological

- 41 -

activity, including Avr9 from *Cladosporium fulvum* (Van den Ackervecken, et al., "The AVR9 Race-Specific Elicitor of *Cladosporium fulvum* is Processed by Endogenous and Plant Proteases," Pl. Physiol. 103:91-96 (1993), which is hereby incorporated by reference), Pep-13 of *Phytophthora megasperma* (Nürnberg, et al.,
5 "High Affinity Binding of a Fungal Oligopeptide Elicitor to the Parsley Plasma Membranes Triggers Multiple Defence Responses," Cell, 78:449-460 (1994), which is hereby incorporated by reference), and harpin_{Pss} of *P. syringae* pv. *syringae* (Alfano, et al., "Analysis of the Role of the *Pseudomonas syringae* HrpZ harpin in Elicitation of the Hypersensitive Response in Tobacco Using Functionally Nonpolar *hrpZ*
10 Deletion Mutations, Truncated HrpZ Fragments, and *hrmA* Mutations," Mol. Microbiol. 19:715-728 (1996), which is hereby incorporated by reference).

Expression of truncated harpin fragments and proteolysis of full-length harpins showed that two distinct fragments retain HR-eliciting activity. The primary sequence of each active fragment show no discernable similarity with each other, or
15 with other elicitor-active peptides. However, the sites of cleavage by trypsin and endoproteinase Glu-C suggest portions of each fragment required for activity. It would be of interest to alter specifically the amino acid residues at or near these cleavage sites to determine whether HR-eliciting activity is altered or lost. Additionally, harpin_{Ea} P64 and P68 demonstrate distinct hydrophobicity during
20 reverse-phase HPLC (Figure 6), and they correspond to a hydrophobic peak in a Kyte-Doolittle plot (Figure 9). The role of this putative hydrophobic domain could be tested by mutagenesis, or by synthesis of altered peptides. However, the fact that multiple fragments independently possess HR-eliciting activity complicates analysis of full-length proteins.

25 This finding, that fragments of the protein retain HR-eliciting activity, also allowed (at least) two apoplastic proteinase activities, which are distinct from intracellular plant proteinases, to be defined. Two apoplastic plant proteinases (from soybean) have been studied in some detail. SMEP, a metalloproteinase (Huangpu, et al., "Purification and Developmental Analysis of an Extracellular Proteinase From
30 Young Leaves of Soybean," Plant Physiol 108:969-974 (1995); McGeehan, et al., "Sequencing and Characterization of the Soybean Leaf Metalloproteinase," Plant Physiol. 99:1179-1183 (1992), which are hereby incorporated by reference) sensitive

- 42 -

to EDTA, is thought to cleave at G/L and G/I. Interestingly, although there are 19 potential SMEP cleavage sites in the intact harpin_{Ea}, only one of them is located within fragments P91 and P95, and none are within fragments P64 and P68 (Figure 7). P91 and P95 thus may represent partial digestion products of a SMEP-like proteinase in the tobacco apoplast. The other studies soybean apoplastic proteinase, SLAP, a

5 sulfhydryl proteinase (Huangpu, et al., "Purification and Developmental Analysis of an Extracellular Proteinase From Young Leaves of Soybean," Plant Physiol. 108:969-974 (1995), which is hereby incorporated by reference) sensitive to p-chloromercuribenzoic acid (pCMB). Several lines of evidence suggest that multiple

10 proteolytic activities in the IF are degrading harpin_{Ea}. PMSF, a serine protease inhibitor, decreases but does not entirely block harpin_{Ea} degradation (Figure 5C); no single proteinase inhibitor tested blocks harpin degradation, and the cleavage sites after residues 109, 136, 200, and 204 are dissimilar. Endoproteinase Glu-C does not abolish activity of full-length harpin, but does abolish activity of P91 and P95 (and

15 presumably P64 and P68); trypsin abolishes the activity of P64 and P68 (and presumably P91 and P95). These final digests suggest specific portions of each distinct HR-eliciting peptide which are presumably necessary for its activity, as mentioned previously.

The apoplastic activities degrade harpin without destroying its HR-eliciting

20 ability, in contrast to intracellular proteolytic activities present in a ground leaf-extract, which abolish activity. This raises a number of intriguing questions, e.g., does the plant use these harpin fragments as elicitor-signals? The timing of the HR was examined when full length harpin and harpin predigested by tobacco intercellular fluid were each infiltrated into tobacco leaves. The HR elicited by each preparation

25 occurred 12-18 hours after infiltration. Co-infiltration of proteinase inhibitors into tobacco leaf panels along with harpin also had no effect on harpin's HR eliciting activity, although limited proteolytic degradation cannot be ruled out in this case, particularly since it seems that at least two, and perhaps more, apoplastic proteinases are present in tobacco. Because predigested harpin elicited the HR no faster than

30 undigested protein, proteolytic digestion seems unlikely to be a rate-limiting step required for HR to occur. The role of these apoplastic proteinases which are able to hydrolyze harpin partially, yet unable to abolish harpin's HR-eliciting activity on

- 43 -

tobacco, remains unclear. Salzer et al., "Rapid Reactions of Spruce Cells to Elicitors Released From the Ectomycorrhizal Fungus *Hebeloma crustuliniforme* and Inactivation of These Elicitors by Extracellular Spruce Cell Enzymes," Planta 198:118-126 (1996), which is hereby incorporated by reference, have noted that

5 spruce (*Picea abies* (L.) Karst.) modulates the level of fungal cell wall elicitors released by the ectomycorrhizal fungus *Hebeloma crustuliniforme* by inactivating these molecules in the apoplast. They propose that *Picea* controls the elicitor level as part of its symbiotic interaction with the fungus. Similarly, PGIP of *Phaseolus vulgaris* has been suggested to modulate the level of elicitor-active

10 oligogalacturonides present during the plant-parasite interaction in bean (Desiderio, et al., "Polygalacturonase, PGIP, and Oligogalacturonides in Cell-Cell Communication," Biochem. Sci. Trans. 22:394-397 (1994), which is hereby incorporated by reference). Perhaps the retention of HR-eliciting activity by harpin fragments contributes to the ability of plants to recognize the presence of a pathogen. In this regard, it would be

15 interesting to explore whether transgenic host and non-host plants, engineered for apoplastic expression of a harpin activity-degrading proteinase, would exhibit reduced or increased sensitivity to *E. amylovora*, compared to non-engineered plants.

Despite numerous attempts, only a handful of truncated derivatives of harpin_{Ea} and harpin_{Ear} were successfully expressed from portions of *hrpN*. Problems with

20 protein stability were evident in that several truncated harpins were unstable and difficult to purify. Additionally, expression of truncated harpins may be deleterious to bacteria. Truncated harpin_{Ea}C31 was, however, stable and easily purified, but not secreted, suggesting that C-terminal sequences are involved in harpin secretion. Unfortunately, the presence of vector-encoded amino acids in this protein complicates

25 this conclusion. All attempts to clone β -galactosidase-harpin fusion proteins have been unsuccessful, as were attempts to clone and express *hrpN* downstream of the *lacZ* promoter in several commonly used vectors such as pBluescript. Expression of such constructs is evidently deleterious to bacterial strains.

Wei, et al., "Harpin, Elicitor of the Hypersensitive Response Produced By the

30 Plant Pathogen *Erwinia amylovora*," Science, 257:85-88 (1992), which is hereby incorporated by reference, reported previously that BLAST searches indicated harpin_{Ea} possessed slight similarity to several other glycine-rich proteins, including

- 44 -

keratins and glycine-rich cell wall proteins. However, this is thought to be due to the high glycine content of harpin_{Ea}, and as such does not suggest a role for harpin_{Ea}. Examination of N-terminal sequences from several HR-eliciting proteins produced by phytopathogenic bacteria (Figure 8) yielded some potential similarities. However, the region in question is quite short. The region of putative primary sequence similarity is limited to the first 26 residues at the N-terminus, and its role remains unclear. Surprisingly, *E. carotovora* harpin_{Ecc} appears more similar to the harpins from *E. amylovora* and *E. stewartii* than to that from *E. chrysanthemi*, to which it is more closely related with respect to its taxonomic position as well as its mechanism of pathogenicity (i.e. soft-rots). In addition, although primary sequence similarity is strongest only in the C-terminal third of each protein, the *Erwinia* harpins possess broadly similar hydrophobicity profiles along their entire lengths (Figure 9). Based on its hydrophobicity profile, Alfano, et al., "Analysis of the Role of the *Pseudomonas syringae* HrpZ harpin in Elicitation of the Hypersensitive Response in Tobacco Using Functionally Nonpolar *hrpZ* Deletion Mutations, Truncated HrpZ Fragments, and *hrmA* Mutations," Mol. Microbiol. 19:715-728 (1996), which is hereby incorporated by reference, speculated that harpin_{Pss} may have an amphiphilic nature. However, the *Erwinia* harpins' profiles do not match that of harpin_{Pss}.

Recently, a number of other secreted glycine-rich pathogenicity associated proteins, elicitors of the HR or other plant-defense responses, have been described from other plant pathogenic bacteria and fungi (Boller, "Chemoperception of Microbial Signals in Plant Cells," Ann. Rev. Plant Physiol. Plant Molec. Biol. 46:189-214 (1996), which is hereby incorporated by reference), including *Phytophthora megasperma* (Ballieul, et al., "A New Elicitor of the Hypersensitive Response in Tobacco: a Fungal Glycoprotein Elicits Cell Death, Expression of Defence Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant Journal 8:551-560 (1995); Nürnburger, et al., "High Affinity Binding of a Fungal Oligopeptide Elicitor to the Parsley Plasma Membranes Triggers Multiple Defence Responses," Cell 78:449-460 (1994), which are hereby incorporated by reference), and *Magnaporthe grisea* (Sweigard, et al., "Identification, Cloning, and Characterization of PWL2, a Gene For Host Species Specificity in the Rice Blast Fungus," Plant Cell 7:1221-1233 (1995), which is hereby incorporated by reference).

- 45 -

Proteinaceous HR-elicitors have also now been described from *Phytophthora* *secalis* (Rohe, et al., "The Race-Specific Elicitor, NIP1, From the Barley Pathogen, *Rhynchosporium secalis*, Determines Avirulence on Host Plants of the *Rrs1* Resistance Genotype," EMBO Journal 14:4168-4177 (1995) which is hereby
5 incorporated by reference, while *P. infestans* (Pieterse, et al., "Structure and Genomic Organization of the *ipiB* and *ipiO* Gene Clusters of *Phytophthora infestans*," Gene, 138:67-77 (1994), which is hereby incorporated by reference) produces a glycine-rich pathogenicity-associated family of proteins of unknown function. Because the
10 primary amino acid sequence of each elicitor protein or peptide fragment shows no obvious similarity to that of the others, it is unclear whether they interact with the same target on or in the plant cell, plasma membrane, or cell wall. In that regard, it might be of interest to test whether any one of these molecules inhibits the action of other(s). The increasing availability of peptides such as Pep13, Avr9, P68, and harpin_{Ea} C305 with plant-defense response-eliciting activity (HR and otherwise)
15 should enable precise probing of their targets on or in plant cells, as well as determination of whether their mechanisms of activity are similar, distinct, or overlapping.

20 Example 14 - Bacterial strains and plasmids

Escherichia coli stains used in the following examples include DH5 α and BL21(DE3) purchased from Gibco BRL and Stratagene, respectively. The pET28(b) vector was purchased from Novagen. Eco DH5 α /2139 contained the complete hrpN gene. The 2139 construct was produced by D. Bauer at the Cornell University. The
25 hrpN gene was cleaved from the 2139 plasmid by restriction enzyme digestion with HindIII, then purified from an agarose gel to serve as the DNA template for PCR synthesis of truncated hrpN clones. These clones were subsequently inserted into the (His)₆ vector pET28(b) which contained a Kan^r gene for selection of transformants.

30 Example 15 - DNA Manipulation

Restriction enzymes were obtained from Boehringer Mannheim or Gibco BRL. T4 DNA ligase, Calf Intestinal Alkaline Phosphatase (CIAP), and PCR

- 46 -

SupermixTM were obtained from Gibco BRL. The QIAprep Spin Miniprep Kit, the Qiagen Plasmid Mini Kit, and the QIAquick PCR Purification Kit were purchased from Qiagen. The PCR primers were synthesized by Lofstrand Labs Limited (Gaithersburg, MD). The oligopeptides were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). All DNA manipulations such as plasmid isolation, restriction enzyme digestion, DNA ligation, and PCR were performed according to standard techniques (molecular cloning) or protocols provided by the manufacturer.

Example 16 - Fragmentation of *hrpN* gene

10 A series of N-terminal and C-terminal truncated *hrpN* genes and internal fragments were generated via PCR (Fig. 10). The full length *hrpN* gene was used as the DNA template and 3' and 5' primers were designed for each truncated clone (Fig. 11). The 3' primers contained in the NdeI enzyme cutting site which contained
15 the start codon ATG (Methionine) and the 5' primers contained the stop codon TAA and a HindIII enzyme cutting site for ligation into the pET28(b) vector. PCR was carried out in 0.5 ml tubes in a GeneAmpTM 9600 or 9700. 45 µl of SupermixTM were mixed with 20 pmoles of each pair of DNA primers, 10 ng of full length harpin DNA, and diH₂O to a final volume of 50 µl. After heating the mixture at 95°C for 2 min, the
20 PCR was performed for 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min. The PCR products were verified on a 6% TBE gel (Novex). Amplified DNA was purified with the QIAquick PCR purification kit, digested with Nde I and Hind III at 37°C for 5 hours, extracted once with phenol:chloroform:isoamylalcohol (25:25:1) and precipitated with ethanol. 5 µg of pET28(b) vector DNA were digested
25 with 15 units of Nde I and 20 units of Hind III at 37°C for 3 hours followed with CIAP treatment to reduce the background resulting from incomplete single enzyme digestion. Digested vector DNA was purified with the QIAquick PCR purification kit and directly used for ligation. Ligation was carried out at 14-16°C for 5-12 hours in a 15 µl mixture containing ca. 200 ng of digested pET28(b), 30 ng of targeted PCR
30 fragment, and 1 unit T4 DNA ligase. 5 - 7.5 µl of ligation solution were added to 100 µl of DH5α competent cells in a 15 ml falcon tube and incubated on ice for 30 min. After a heat shock at 42°C for 45 seconds, 0.9 ml SOC solution or 0.45 ml LB media

- 47 -

were added to each tube and incubated at 37°C for 1 hour. 20, 100, and 200 µl of transformed cells were placed onto LB agar with 30 µg/ml of kanamycin and incubated at 37°C overnight. Single colonies were transferred to 3 ml LB-media and incubated overnight at 37°C. Plasmid DNA was prepared from 2 ml of culture with the QIAprep Miniprep kit. The DNA from the transformed cells was analyzed by restriction enzyme digestion or partial sequencing to verify the success of the transformations. Plasmids with the desired DNA sequence were transferred into the BL21 strain using the standard chemical transformation method as indicated above. A clone containing the full length harpin protein in the pet28(b) vector was generated as a positive control, and a clone with only the pET28(b) vector was generated as a negative control.

Example 17 - Expression of harpin truncated proteins

Escherichia coli BL21(DE3) strains containing the hrpN clones were grown in Luria broth medium (g/L Difco Yeast extract, 10 g/L Difco Tryptone, 5g/L NaCl, and 1 mM NaOH) containing 30 µg/ml of kanamycin at 37°C overnight. The bacteria were then inoculated into 100 volumes of the same medium and grown at 37°C to an OD₆₂₀ of 0.6-0.8. The bacteria were then inoculated into 250 volumes of the same medium and grown at 37°C to an OD₆₂₀ of ca. 0.3 or 0.6-0.8. One milli molar IPTG was then added and the cultures grown at 19°C overnight (ca. 18 hours). Not all of the clones were successfully expressed using this strategy. Several of the clones had to be grown in Terrific broth (12 g/L Bacto Tryptone, 24 g/L Bacto yeast, 0.4% glycerol, 0.17 M KH₂PO₄, and 0.72 K₂HPO₄), and/or grown at 37°C after IPTG induction, and/or harvested earlier than overnight (Table 2).

Table 2: Expression of harpin truncated proteins

Fragment	amino acids (SEQ. ID. No. 23)	Growth medium	Induction O.D.	Expression temp.	Harvest time
1 (+ control)	1-403	LB	ca. 0.3 or 0.6-0.8	19°C or 25°C	16-18 hr
2 (+ control)	-	LB and TB	ca. 0.3 or 0.6-0.8	19 C and 37 C	16-18 hr
3	105-403	LB	0.6-0.8	19°C	16-18 hr
4	169-403	TB	ca. 0.3	19°C	16-18 hr
5	210-403	LB or M9ZB	0.6-0.8	19°C	16-18 hr
6	257-403	LB or M9ZB	0.6-0.8	19°C	16-18 hr
7	343-403	LB	ca. 0.3	19°C	5 hr
8	1-75	TB	ca. 0.3	37°C	16-18 hr
9	1-104	TB	ca. 0.3	37°C	16-18 hr
10	1-168	TB	ca. 0.3	37°C	16-18 hr
11	1-266	LB	ca. 0.3	37°C	4 hr
12	1-342	LB	0.6-0.8	19°C	16-18 hr
13	76-209	LB	ca. 0.3	37°C	5 hr
14	76-168	TB or LB	ca. 0.3	37°C	3 hr or 16-18 hr
15	105-209	M9ZB	ca. 0.3	37°C	3 hr
16	169-209	no expression			
17	105-168	LB	ca. 0.3	37°C	3-5 hr
18	99-209	LB	ca. 0.3	37°C	3 hr
19	137-204	LB	ca. 0.3	37°C	3 hr
20	137-180	LB	ca. 0.3	37°C	16-18 hr.
21	105-180	LB	ca. 0.3	37°C	3 hr
22	150-209	no expression			
23	150-180	no expression			

- 5 General expression method: *Escherichia coli* BL21(DE3) strains containing the hrpN subclones were grown in Luria broth medium (5g/L Difco Yeast extract, 10 g/L Difco Tryptone, 5g/L NaCl, and 1 mM NaOH) containing 30 µg/ml of kanamycin at 37°C overnight. The bacteria were then inoculated into 100 volumes of the same medium and grown at 37°C to an OD₆₂₀ of 0.6-0.8. The bacteria were then inoculated into 250 volumes of the growth medium and grown at 37°C to a specific induction OD₆₂₀. One milli molar IPTG was then added and the cultures grown at an optimal temperature for protein expression, and harvested at a particular time for recovery of the highest level of protein.
- 10

Example 18 - Small scale purification of harpin truncated proteins (verification of expression)

15

- A 50 ml culture of a hrpN clone was grown as above to induce expression of the truncated protein. Upon harvesting of the culture, 1.5 ml of the cell suspension were centrifuged at 14,000 rpm for 5 minutes, re-suspended in urea lysis buffer (8 M urea, 0.1 M Na₂HPO₄, and 0.01 M Tris -- pH 8.0), incubated at room temperature for 10 minutes, then centrifuged again at 14,000 rpm for 10 minutes, and the supernatant
- 20

- 49 -

saved. A 50 µl aliquot of a 50% slurry of an equilibrated (His)₆-binding nickel agarose resin was added to the supernatant and mixed at 4°C for one hour. The nickel agarose was then washed three times with urea washing buffer (8 M urea, 0.1 M Na₂HPO₄, and 0.01 M Tris -- pH 6.3), centrifuging at 5,000 rpm for five minutes
5 between washings. The protein was eluted from the resin with 50 µl of urea elution buffer (8 M urea, 0.1 M Na₂HPO₄, 0.01 M Tris, and 0.1 M EDTA -- pH 6.3). The eluate was run on a 4-20%, a 16%, or a 10-20% Tris-Glycine pre-cast gel depending upon the size of the truncated protein to verify the expression.

Example 19 - Induction of HR in tobacco

10

A 1.5 ml aliquot from the 50 ml cultures grown for small scale purification of the truncated proteins was centrifuged at 14,000 rpm for four minutes and re-suspended in an equal volume of 5 mM potassium phosphate buffer, pH 6.8. The cell suspension was sonicated for ca. 30 seconds then diluted 1:2 and 1:10 with
15 phosphate buffer. Both dilutions plus the neat cell lysate were infiltrated into the fourth to ninth leaves of 10-15 leaf tobacco plants by making a hole in single leaf panes and infiltrating the bacterial lysate into the intercellular leaf space using a syringe without a needle. The HR response was recorded 24-48 hr post infiltration. Tobacco (*Nicotiana tabacum* v. Xanthi) seedlings were grown in an environmental
20 chamber at 20-25°C with a photoperiod of 12-h light /12-h dark and ca. 40% RH. Cell lysate was used for the initial HR assays (in order to screen the truncated proteins for HR activity) as the small scale urea purification yielded very little protein which was denatured due to the purification process.

Example 20 - Large scale native purification of harpin truncated proteins for comprehensive biological activity assays

Six 500 ml cultures of a hrpN clone were grown as described earlier to induce expression of the truncated protein. Upon harvesting of the culture the cells were
30 centrifuged at 7,000 rpm for 5 minutes, re-suspended in imidazole lysis buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris) plus Triton X-100 at 0.05% and lysozyme at 0.1 mg/ml, and incubated at 30°C for 15 minutes, sonicated for two minutes, then centrifuged again at 15,000 rpm for 20 minutes, and the supernatant was saved. A 4

- 50 -

ml aliquot of a 50% slurry of an equilibrated (His)₆-binding nickel agarose resin was added to the supernatant and mixed at 4°C for ca. four hours. The nickel agarose was then washed three times with imidazole washing buffer (20 mM imidazole, 0.5 M NaCl, and 20 mM Tris), centrifuging at 5,000 rpm for five minutes between washings, then placed in a disposable chromatography column. The column was centrifuged at 1100 rpm for one minute to remove any residual wash buffer and then the protein was eluted from the resin with 4 ml of imidazole elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris) by incubating the column with the elution buffer for ten minutes at room temperature and then centrifuging the column at 1100 rpm for one minute. The eluate was run on a 4-20%, a 16%, or a 10-20% Tris-Glycine pre-cast gel depending upon the size of the truncated protein to verify the expression. The concentration of the proteins was determined by comparison of the protein bands with a standard protein in the Mark 12 molecular weight marker.

Example 21 - Large scale urea purification of harpin truncated proteins for comprehensive biological activity assay

The procedure was the same as the large scale native purification except that urea lysis buffer, washing buffer, and elution buffer were used, and the cells were not sonicated as in the native purification. After purification, the protein was renatured by dialyzing against lower and lower concentrations of urea over an eight hour period, then dialyzing overnight against 10 mM Tris/20 mM NaCl. The renaturing process caused the N-terminal proteins to precipitate. The precipitated 1-168 protein was solubilized by the addition of 100 mM Tris-HCl at pH 10.4 then heating the protein at 30°C for ca. one hour. The concentration of the protein was determined by comparison of the protein bands with a standard protein in the Mark 12 molecular weight marker. The 1-75 and 1-104 protein fragments were not successfully solubilized using this strategy so they were sonicated in 100 mM Tris-HCl at pH 10.4 to solubilize as much of the protein as possible and expose the active sites of the protein for the biological activity assays.

Example 22 - Expression of harpin truncated proteins

The small scale expression and purification of the fragment proteins was done to screen for expression and HR activity (Table 3).

5

Table 3

Expression and HR activity of harpin truncated proteins (small scale screening)

Fragment #	Amino Acids (SEQ. ID. No. 23)	Expression	HR activity
1(+control)	1-403	+	+
2(- control)	-	background protein only	-
3	105-403	+	+
4	169-403	+	-
5	210-403	+	-
6	267-403	+	-
7	343-403	+/-	-
8	1-75	+	-
9	1-104	+	+/-
10	1-168	+	+
11	1-266	+	+
12	1-342	+	+
13	76-209	+	+
14	76-168	+	-
15	105-209	+	+
16	169-209	-	-
17	105-168	+	-
18	99-209	+	+
19	137-204	+	+
20	137-180	+	+
21	105-180	+	+
22	150-209	-	-
23	150-180	-	-

- 10 All of the cloned fragment proteins were expressed to a certain degree except for three small fragments (amino acids 169-209, 150-209, and 150-180). The fragments were expressed at varying levels. Fragments 210-403 and 267-403 were expressed very well, yielding a high concentration of protein from a small scale purification, resulting in a substantial protein band on SDS gel electrophoresis. Other fragments (such as
- 15 a.a. 1-168 and 1-104) produced much less protein, resulting in faint protein bands upon electrophoresis. It was difficult to determine whether fragment 343-403, the smallest C-terminal protein, was expressed, as there were several background proteins apparent on the gel, in addition to the suspected 343-403 protein. The positive and

- 52 -

negative control proteins, consisting of the full length harpin protein and only background proteins, respectively, were tested for expression and HR activity as well.

The large scale expression and purification of the fragment proteins was done to determine the level of expression and titer of the HR activity (Table 4).

5

Table 4

Expression level and HR titer of harpin truncated proteins (large sale purification)

Fragment #	Amino acids (SEQ. ID. No. 23)	Expression	HR titer
1(+ control)	1-403	3.7 mg/ml	5-7 µg/ml
2(- control)	-	-	1:2 dilution
4	169-403	2 mg/ml	-
5	210-403	5 mg/ml	-
6	267-403	4 mg/ml	-
7	343-402	200µg/ml	-
8	1-75	50µg/ml	-
9	1-104	50µg/ml	3 µg/ml (1:16 dilution)
10	1-168	1 mg/ml	1 µg/ml
13	76-209	2.5 mg/ml	5 µg/ml
14	76-168	2 mg/ml	-
15	105-209	5 mg/ml	5-10µg/ml
17	105-168	250µg/ml	-
19	137-204	3.6 mg/ml	3.5 µg/ml
20	137-180	250 µg/ml	16 µg/ml

10

Not all of the proteins were expressed in large scale due to time constraints. The truncated proteins deemed to be the most important in characterizing harpin were chosen. The positive control (full length harpin) was expressed in a relatively high level at 3.7 mg/ml. All of the C-terminal proteins were expressed at relatively high levels from 2-5 mg/ml, except for fragment 343-403 as discussed earlier. The N-terminal fragments were expressed very well also, however, during the purification process, the protein precipitated and very little was resolubilized. The concentrations in Table 3 reflect only the solubilized protein. The internal fragments were expressed in the range of 2-3.6 mg/ml. It was extremely difficult to determine the concentration of fragment 105-168 (it was suspected that the concentration was much higher than indicated), as the protein bands on the SDS gel were large, but poorly stained. The negative control contained several background proteins as expected, but no obviously induced dominant protein.

15

20

Example 23 - Induction of HR in tobacco

The full length positive control protein elicited HR down to only 5-7 µg/ml. The negative control (pET 28) imidazole purified "protein" - which contained only
5 background proteins - elicited an HR response down to the 1:2 dilution, which lowered the sensitivity of the assay as the 1:1 and 1:2 dilutions could not be used. This false HR was likely due an affinity of the imidazole used in the purification process to bind to one or several of the background proteins, thereby not completely dialyzing out. Imidazole at a concentration of ca. 60 mM did elicit a false HR
10 response.

One definitive domain encompassed a small internal region of the protein from a.a. 137-180 (SEQ. ID. No. 23), a mere 44 a.a, is identified as the smallest HR domain. The other potential HR domain is thought to be located in the N-terminus of the protein from a.a. 1-104 (possibly a.a. 1-75) (SEQ. ID. No. 23). It was difficult to
15 confirm or narrow down the N-terminus HR domain due to the difficulties encountered in purifying these fragment proteins. The N-terminus fragment proteins had to be purified with urea as no protein was recovered when the native purification process was used. Consequently, these proteins precipitated during the renaturing process and were difficult or nearly impossible to get back into solution, thereby
20 making it hard to run the proteins through the HR assay, as only soluble protein is able to elicit HR. Difficulty narrowing the N-terminus HR domain was only compounded by the fact that the negative control elicited false HR at the low dilution levels thereby reducing the sensitivity of the assay.

The internal domain proteins elicited an HR response between 5 and 10 µg/ml
25 of protein like the positive control, and the N-terminus domain proteins elicited an HR response between 1 and 3 µg/ml, lower than the positive control.

Surprisingly, when the internal HR domain was cleaved between a.a. 168 and 169 (fragments 76-168 and 105-168) (SEQ. ID. No. 23) the fragment lost its HR activity. This suggests that the HR activity of fragment 1-168 (SEQ. ID. No. 23)
30 should not be attributed to the internal HR domain, but rather to some other domain, leading to the assumption that there was likely a second HR domain to be found in the N-terminal region of the protein. However, as discussed earlier it was difficult to confirm this assumption.

- 54 -

The harpin C-terminus (a.a. 210-403 (SEQ. ID. No. 23)) did not contain an HR domain. It did not elicit HR at a detectable level using the current HR assay. Even the large C-terminal fragment from a.a. 169-403 (SEQ. ID. No. 23) did not elicit HR even though it contained part of the internal HR domain. As stated above, the
5 protein between a.a. 168 and 169 (SEQ. ID. No. 23) causes a loss of HR activity.

Because some of the small cloned proteins with 61 a.a. or less were not expressed, several oligopeptides were synthesized with 30 a.a. to narrow down the functional region of the internal HR domain. The oligopeptides were synthesized within the range of a.a. 121-179 (SEQ. ID. No. 23). However, these oligos did not
10 elicit the HR response. It was not expected that there would be an HR response from oligos 137-166, 121-150, and 137-156 (SEQ. ID. No. 23) as these fragments did not contain the imperative amino acids 168 and 169 (SEQ. ID. No. 23). It was expected that the oligo 150-179 (SEQ. ID. No. 23) would elicit an HR response. It is possible that 30 a.a. is too small for the protein to elicit any activity due to a lack of folding
15 and, therefore, a lack of binding or that during the synthesis of the peptides important amino acids were missed (either in the process, or simply by the choice of which 30 amino acids to synthesize) and, therefore, the fragments would not be able to elicit HR. It is also possible, although unlikely, that these small proteins would have undergone some form of post-translational modification within the *E.coli* cell that
20 they did not contain when synthesized and, therefore, were not able to elicit an HR response.

Example 24 – Biological Activity of HR Inducing Fragments

25 The two N-terminal harpin fragments spanning nucleotides 1-104 and nucleotides 1-168 of the nucleic acid of SEQ. ID. No. 24 were effective at inducing resistance of tobacco against TMV, in a similar manner as the full length harpin protein. The internal fragments spanning nucleotides 76-209 and nucleotides 105-209 of the nucleic acid of SEQ. ID. No. 24 were also effective at inducing TMV
30 resistance. In addition, these same four fragments conferred plant growth enhancement ("PGE") in tomato increasing the height of the plants from 4-19% taller than the buffer control plants. The full length harpin protein induced growth

- 55 -

enhancement of 6% greater than the buffer. The negative control did not induce TMV resistance or growth enhancement.

Table 5

5 TMV resistance and PGE activity of HR inducing fragments derived from harpin

Fragment #	Amino acids (SEQ. ID. No. 23)	HR activity	TMV resistance	PGE ht > buffer
1 (+ control)	1-403	+	+	6%
2 (- control)	-	-	-	-2%
9	1-104	+	+	4-8%
10	1-168	+	+	5-13%
13	76-209	+	+	4-18%
15	105-209	+	+	6-19%

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations
10 can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

- 56 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Cornell Research Foundation, Inc. and EDEN Bioscience Corporation
- (ii) TITLE OF INVENTION: HYPERSENSITIVE RESPONSE ELICITOR
FRAGMENTS ELICITING A HYPERSENSITIVE
RESPONSE AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 30
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Nixon, Hargrave, Devans & Doyle LLP
 - (B) STREET: Clinton Square, P.O. Box 1051
 - (C) CITY: Rochester
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 14603
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/048,109
 - (B) FILING DATE: 30-MAY-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Goldman, Michael L.
 - (B) REGISTRATION NUMBER: 30,727
 - (C) REFERENCE/DOCKET NUMBER: 19603/1302
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (716) 263-1304
 - (B) TELEFAX: (716) 263-1600

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- 57 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAATTCAT ATGAGTCTGA ATACAAGTGG G

31

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGAATTCAT ATGGGCGGTG GCTTAGGCGG T

31

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCATATGTC GAACGCGCTG AACGATATG

29

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGAATTCAT ATGTTAGGCG GTTCGCTGAA C

31

(2) INFORMATION FOR SEQ ID NO:5:

- 58 -

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCATATGCT GAACACGCTG GGCTCGAAA

29

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCATATGTC AACGTCCCAA AACGACGAT

29

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGCATATGTC CACCTCAGAC TCCAGCG

27

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- 59 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAATTCAT ATGCAAAGCC TGTTTGGTGA TGGG

34

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGAATTCAT ATGGGTAATG GTCTGAGCAA G

31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGAATTCAT ATGAAAGCGG GCATTCAGGC G

31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGAATTCAT ATGACACCAG CCACTATGGA GCAG

34

- 60 -

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCAAGCTTAA CAGCCACCA CCGCCCATCA T

31

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCAAGCTTAA ATCGTTCAGC GCGTTCGACA G

31

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCAAGCTTAA TATCTCGCTG AACATCTTCA GCAG

34

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- 61 -

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCAAGCTTAA GGTGCCATCT TGCCCATCAC

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCAAGCTTAA ATCAGTGACT CCTTTTTTAT AGGC

34

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCAAGCTTAA CAGGCCCGAC AGCGCATCAG T

31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCAAGCTTAA ACCGATACCG GTACCCACGG C

31

- 62 -

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAAGCTTAA TCCGTCGTCA TCTGGCTTGC TCAG

34

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GCAAGCTTAA GCCGCGCCCA GCTTG

25

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 338 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met	Gln	Ile	Thr	Ile	Lys	Ala	His	Ile	Gly	Gly	Asp	Leu	Gly	Val	Ser
1					5				10				15		
Gly	Leu	Gly	Ala	Gln	Gly	Leu	Lys	Gly	Leu	Asn	Ser	Ala	Ala	Ser	Ser
		20						25					30		
Leu	Gly	Ser	Ser	Val	Asp	Lys	Leu	Ser	Ser	Thr	Ile	Asp	Lys	Leu	Thr
		35					40					45			

- 63 -

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
 50 55 60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
 65 70 75 80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
 85 90 95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
 100 105 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
 115 120 125

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
 130 135 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
 145 150 155 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
 165 170 175

Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
 180 185 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
 195 200 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
 210 215 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
 225 230 235 240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
 245 250 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
 260 265 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
 275 280 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
 290 295 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
 305 310 315 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
 325 330 335

Asn Ala

- 64 -

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2141 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCTGA CACCGTTACG	60
GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTGCGCCGCA ATCCGGCGTC	120
GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACCTCA TGATGCAGAT TCAGCCGGGG	180
CAGCAATATC CCGGCATGTT GCGCAGCTG CTCGCTCGTC GTTATCAGCA GCGGCAGAG	240
TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG	300
CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GCGGGGAATG	360
ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CCGACGCGCC	420
CGATCATTAA GATAAAGGCG GCTTTTTTTTA TTGCAAAACG GTAACGGTGA GGAACCGTTT	480
CACCGTCGGC GTCACCTCAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG	540
GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA	600
AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GGCTGGGTGC	660
TCAGGGACTG AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCAGCG TGGATAAACT	720
GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT	780
GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC	840
TTTCGGCAAT GCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA	900
TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC	960
CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC	1020
CCAGGGTAAT ATGAATGCGT TCGGCAGCGG TGTGAACAAC GCACTGTCGT CCATTCTCGG	1080
CAACGGTCTC GGCCAGTCGA TGAGTGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT	1140
GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT	1200
GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCACGTAG ACGGTAACAA	1260

- 65 -

CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA 1320
 TCAGTATCCG GAAATATTTC GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCGAA 1380
 GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG 1440
 CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA 1500
 TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC 1560
 GGCTGTCGTC GCGGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA 1620
 ATCTGTGCTG GCCTGATAAA GCGGAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC 1680
 TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA 1740
~~ACGCACATTT TCCCGTTCAT TCGCGTCGTT AC GCGCCACA ATCGCGATGG CATCTTCCTC 1800~~
 GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC 1860
 CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTTCTATCC GCCCCTTTAG 1920
 CAGATAGATT GCGGTTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG 1980
 GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC 2040
 AAAATAGGGC AGTTTTTTCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG 2100
 GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T 2141

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 403 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met	Ser	Leu	Asn	Thr	Ser	Gly	Leu	Gly	Ala	Ser	Thr	Met	Gln	Ile	Ser
1				5				10					15		
Ile	Gly	Gly	Ala	Gly	Gly	Asn	Asn	Gly	Leu	Leu	Gly	Thr	Ser	Arg	Gln
			20					25				30			
Asn	Ala	Gly	Leu	Gly	Gly	Asn	Ser	Ala	Leu	Gly	Leu	Gly	Gly	Gly	Asn
			35				40					45			

- 66 -

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
 50 55 60
 Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
 65 70 75 80
 Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
 85 90 95
 Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
 100 105 110
 Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
 115 120 125
 Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
 130 135 140
 Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
 145 150 155 160
 Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
 165 170 175
 Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
 180 185 190
 Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
 195 200 205
 Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
 210 215 220
 Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
 225 230 235 240
 Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
 245 250 255
 Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
 260 265 270
 Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
 275 280 285
 Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
 290 295 300
 Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
 305 310 315 320
 Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
 325 330 335

- 67 -

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
 340 345 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
 355 360 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
 370 375 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
 385 390 395 400

Gly Ala Ala

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1288 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA	60
GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT	120
ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG	180
GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG	240
GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG	300
GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA	360
GGACTGTCTGA ACGCGCTGAA CGATATGTTA GGCGGTTTCGC TGAACACGCT GGGCTCGAAA	420
GGCGGCAACA ATACCACTTC AACAAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC	480
TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC	540
CCGATGCAGC AGCTGCTGAA GATGTTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG	600
CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC	660
GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG	720
CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC	780

- 68 -

```

GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGA CTACCAGCAG      840
TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT      900
ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG      960
GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC     1020
CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC     1080
AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC     1140
ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC     1200
GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGCTG ATGCCATTAA CAATATGGCA     1260
CTTGGCAAGC TGGGCGCGGC TTAAGCTT                                     1288

```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 341 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```

Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1              5              10              15
Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
20              25              30
Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Leu Met
35              40              45
Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
50              55              60
Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65              70              75              80
Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85              90              95
Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
100             105             110
Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115             120             125

```

- 69 -

```

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130                               135                               140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145                               150                               155                               160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
                               165                               170                               175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
                               180                               185                               190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
                               195                               200                               205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
210                               215                               220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225                               230                               235                               240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
                               245                               250                               255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
                               260                               265                               270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
275                               280                               285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
290                               295                               300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305                               310                               315                               320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
                               325                               330                               335

Asn Gln Ala Ala Ala
                               340

```

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1026 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 70 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCCTG	60
GTACGTCCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC	120
GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA	180
AAACTGTTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC	240
ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG	300
GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC	360
AAGTCGATGC TCGATGATCT TGTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC	420
GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC	480
AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC	540
GAAACGGCTG CGTTCCGTTT GGCACTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG	600
AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC	660
AACAACCTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC	720
GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA	780
TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCAGAGAC CGGTACGTCG	840
GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG	900
GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT	960
GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA	1020
GCCTGA	1026

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 344 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met	Ser	Val	Gly	Asn	Ile	Gln	Ser	Pro	Ser	Asn	Leu	Pro	Gly	Leu	Gln
1					5					10				15	

- 71 -

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
 20 25 30
 Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
 35 40 45
 Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
 50 55 60
 Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
 65 70 75 80
 Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
 85 90 95
 Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
 100 105 110
 Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
 115 120 125
 Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
 130 135 140
 Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
 145 150 155 160
 Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
 165 170 175
 Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
 180 185 190
 Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
 195 200 205
 Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
 210 215 220
 Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
 225 230 235 240
 Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
 245 250 255
 Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
 260 265 270
 Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
 275 280 285
 Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
 290 295 300

- 72 -

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
 305 310 315 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
 325 330 335

Gln Ser Thr Ser Thr Gln Pro Met
 340

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1035 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC	60
AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC	120
GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC	180
GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC	240
AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC	300
GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA	360
GACCTGGTGA AGCTGCTGAA GGCGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG	420
GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC	480
GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC	540
GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT	600
GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC	660
GGCCCGCAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC	720
CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG	780
ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC	840
GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT	900
GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCCAGATCA TGGATGTGGT GAAGGAGGTC	960

- 73 -

GTCCAGATCC TGCAGCAGAT GCTGGCGGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG 1020
ACGCAGCCGA TGTAA 1035

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Thr Leu Ile Glu Leu Met Ile Val Val Ala Ile Ile Ala Ile Leu Ala
1 5 10 15
Ala Ile Ala Leu Pro Ala Tyr Gln Asp Tyr
20 25

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
1 5 10 15
Leu Leu Ala Met
20

- 74 -

WHAT IS CLAIMED:

1. An isolated fragment of an *Erwinia* hypersensitive response elicitor protein or polypeptide, wherein said fragment elicits a hypersensitive response in
5 plants.
2. An isolated fragment according to claim 1, wherein the hypersensitive response elicitor protein or polypeptide is derived from an *Erwinia amylovora*,
Erwinia carotovora, *Erwinia chrysanthemi*, or *Erwinia stewartii*.
10
3. An isolated fragment according to claim 2, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Erwinia amylovora*.
4. An isolated fragment according to claim 3, wherein the fragment is
15 selected from the group consisting of a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, and an internal fragment of the amino acid sequence of SEQ. ID. No. 23.
- 20 5. An isolated fragment according to claim 4, wherein the fragment is a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning amino acids 105 and 403 of SEQ. ID. No. 23.
6. An isolated fragment according to claim 4, wherein the fragment is an
25 N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning the following amino acids of SEQ. ID. No. 23: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372.
7. An isolated fragment according to claim 4, wherein the fragment is an
30 internal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning the following amino acids of SEQ. ID. No. 23: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.

- 75 -

8. An isolated DNA molecule encoding a fragment according to claim 1.
9. An isolated DNA molecule according to claim 8, wherein the hypersensitive response elicitor protein or polypeptide is derived from an *Erwinia amylovora*, *Erwinia carotovora*, *Erwinia chrysanthemi*, or *Erwinia stewartii*.
10. An isolated DNA molecule according to claim 9, wherein the hypersensitive response elicitor protein or polypeptide is derived from *Erwinia amylovora*.
11. An isolated DNA molecule according to claim 10, wherein the fragment is selected from the group consisting of a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23, and an internal fragment of the amino acid sequence of SEQ. ID. No. 23.
12. An isolated DNA molecule according to claim 10, wherein the fragment is a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning amino acids 105 and 403 of SEQ. ID. No. 23.
13. An isolated DNA molecule according to claim 10, wherein the fragment is an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning the following amino acids of SEQ. ID. No. 23: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372.
14. An isolated DNA molecule according to claim 10, wherein the fragment is an internal fragment of the amino acid sequence of SEQ. ID. No. 23 spanning the following amino acids of SEQ. ID. No. 23: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.

- 76 -

15. An expression system transformed with a DNA molecule according to claim 8.

16. An expression system according to claim 15, wherein said DNA molecule is in proper sense orientation and correct reading frame.

17. A host cell transformed with a DNA molecule according to claim 8.

18. A host cell according to claim 17, wherein the host cell is selected from the group consisting of a plant cell and a bacterial cell.

19. A host cell according to claim 17, wherein the DNA molecule is transformed with an expression system.

20. A transgenic plant transformed with the DNA molecule of claim 8.

21. A transgenic plant according to claim 20, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

22. A transgenic plant according to claim 20, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

23. A transgenic plant seed transformed with the DNA molecule of claim 8.

- 77 -

24. A transgenic plant seed according to claim 23, wherein the plant seed is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, 5 spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

25. A transgenic plant seed according to claim 23, wherein the plant seed 10 is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

26. A method of imparting disease resistance to plants comprising:
applying a fragment of a hypersensitive response elicitor protein or 15 polypeptide, which fragment elicits a hypersensitive response, in a non-infectious form to a plant or plant seed under conditions impart disease resistance.

27. A method according to claim 26, wherein plants are treated during said 20 applying.

28. A method according to claim 26, wherein plant seeds are treated during said applying, said method further comprising:
planting the seeds treated with the fragment of the hypersensitive response elicitor in natural or artificial soil and 25 propagating plants from the seeds planted in the soil.

29. A method of enhancing plant growth comprising:
applying a fragment of a hypersensitive response elicitor protein or polypeptide, which fragment elicits a hypersensitive response, in a non-infectious 30 form to a plant or plant seed under conditions effective to enhance plant growth.

- 78 -

30. A method according to claim 29, wherein plants are treated during said applying.

31. A method according to claim 29, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the fragment of the hypersensitive response elicitor in natural or artificial soil and propagating plants from the seeds planted in the soil.

32. A method of insect control for plants comprising:
applying a fragment of a hypersensitive response elicitor protein or polypeptide, which fragment elicits a hypersensitive response, in a non-infectious form to a plant or plant seed under conditions effective to control insects.

33. A method according to claim 32, wherein plants are treated during said applying.

34. A method according to claim 32, wherein plant seeds are treated during said applying, said method further comprising:

planting the seeds treated with the fragment of the hypersensitive response elicitor in natural or artificial soil and propagating plants from the seeds planted in the soil.

35. A method of imparting disease resistance to plants comprising:
providing a transgenic plant or plant seed transformed with a DNA molecule which encodes a fragment of a hypersensitive response elicitor protein or polypeptide, which fragment elicits a hypersensitive response, and growing the transgenic plant or transgenic plants produced from the transgenic plant seeds under conditions effective to impart disease resistance.

36. A method according to claim 35, wherein a transgenic plant is provided.

37. A method according to claim 35, wherein a transgenic plant seed is provided.

5 38. A method of enhancing plant growth comprising:
 providing a transgenic plant or a plant seed transformed with a DNA
 molecule which encodes a fragment of a hypersensitive response elicitor protein or
 polypeptide, which fragment elicits a hypersensitive response, and
 growing the transgenic plant or transgenic plants produced from the
10 transgenic plant seeds under conditions effective to enhance plant growth.

39. A method according to claim 38, wherein a transgenic plant is provided.

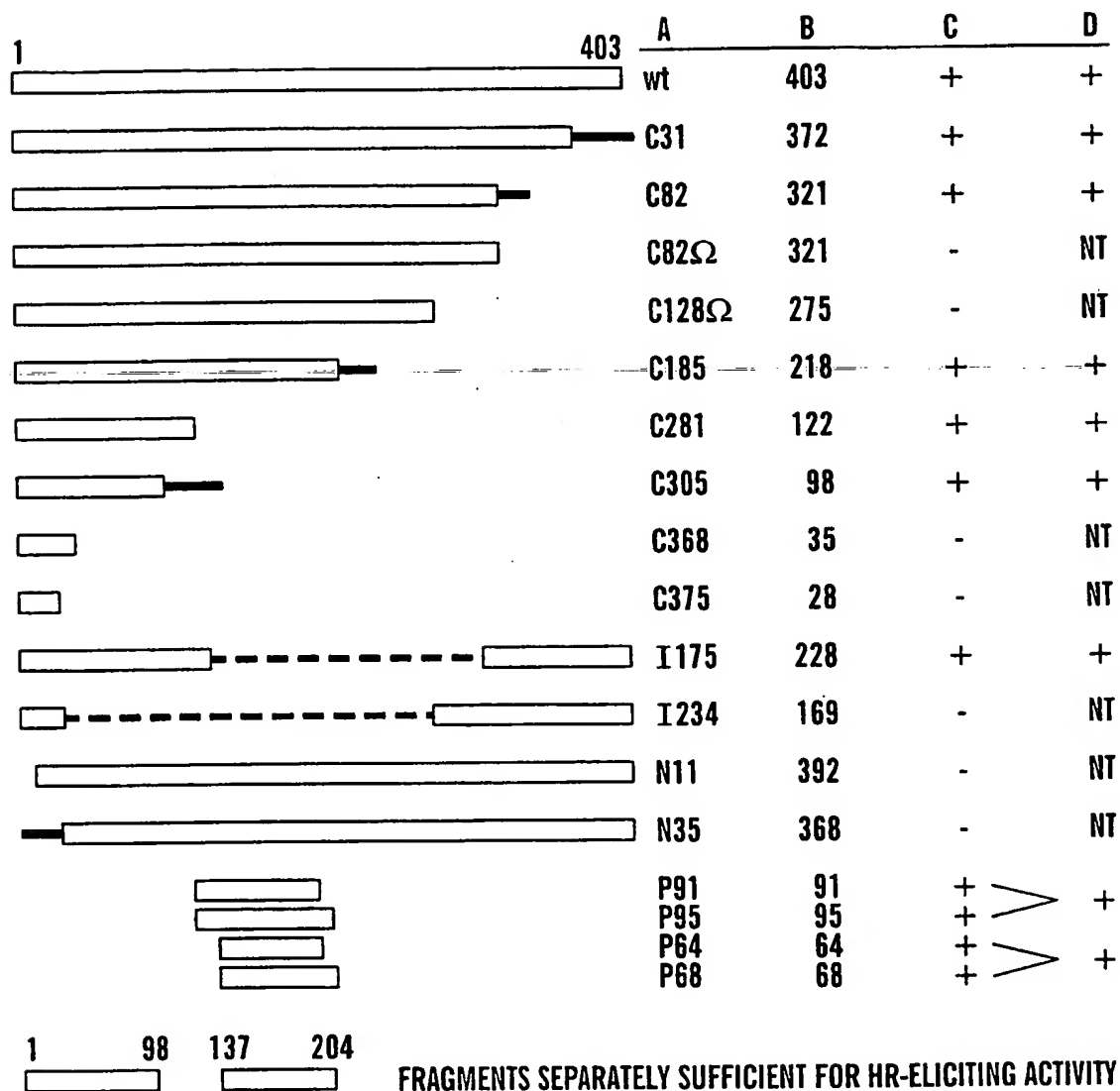
15 40. A method according to claim 38, wherein a transgenic plant seed is provided.

 41. A method of insect control for plants comprising:
 providing a transgenic plant or plant seed transformed with a DNA
20 molecule which encodes a fragment of a hypersensitive response elicitor protein or
 polypeptide, which fragment elicits a hypersensitive response, and
 growing the transgenic plant or transgenic plants produced from the
 transgenic plant seeds under conditions effective to control insects.

25 42. A method according to claim 41, wherein a transgenic plant is provided.

 43. A method according to claim 41, wherein a transgenic plant seed is provided.

1/13

**FIG. 1**

2/13

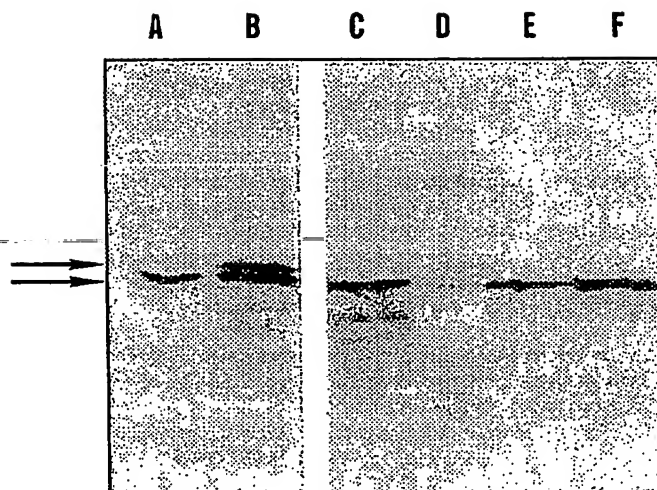


FIG. 2

3/13

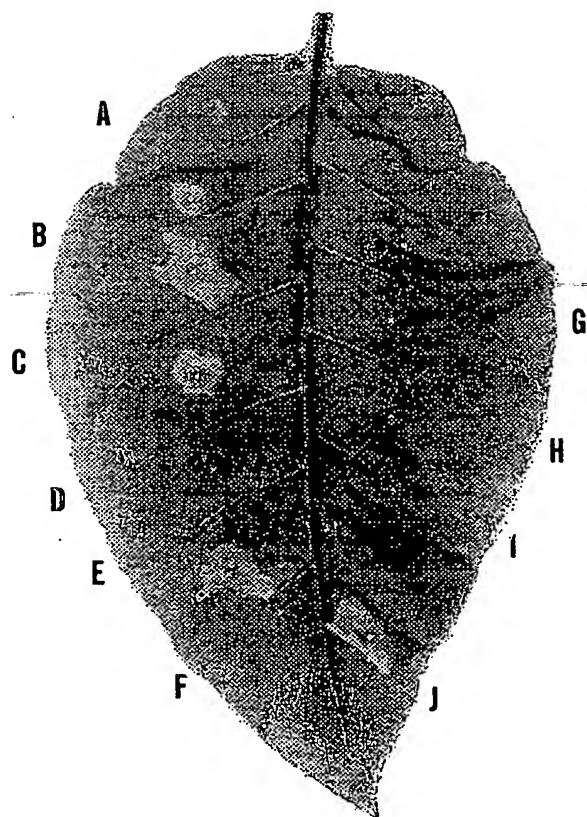


FIG. 3

4/13

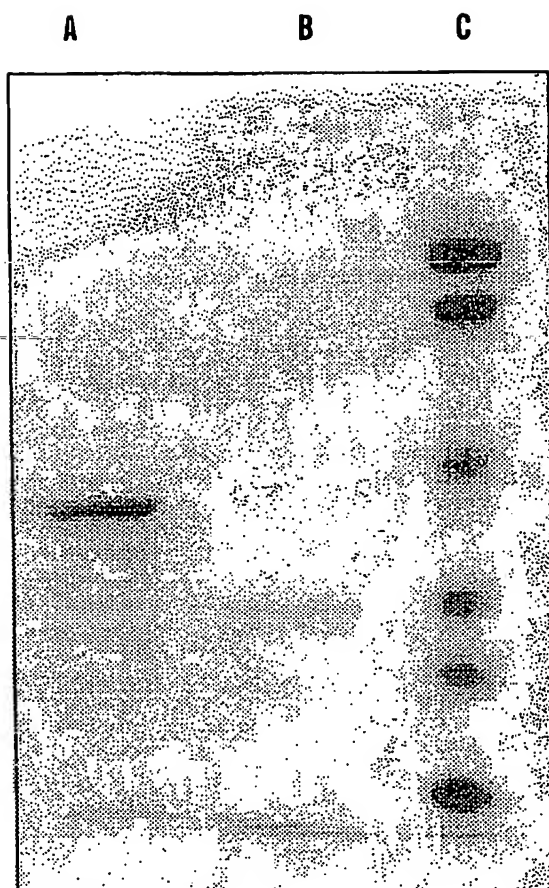
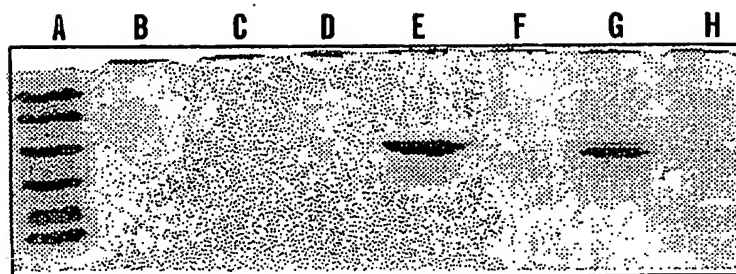
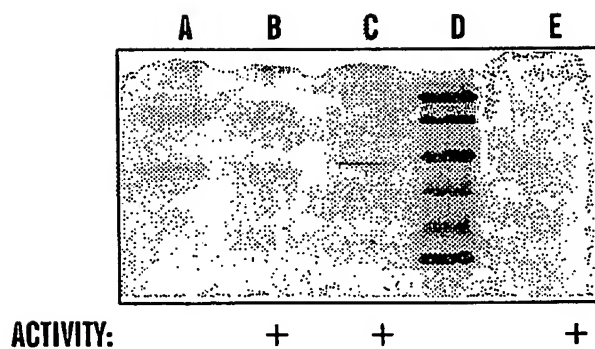


FIG. 4

5/13

**FIG. 5A****FIG. 5B**

6/13

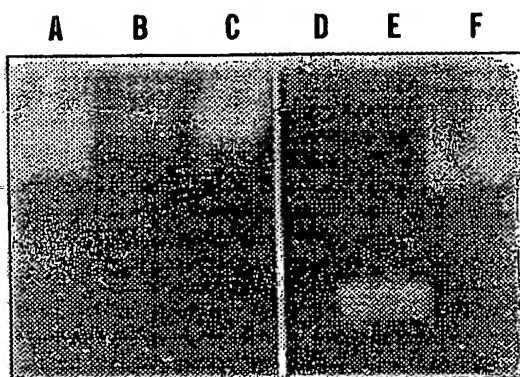
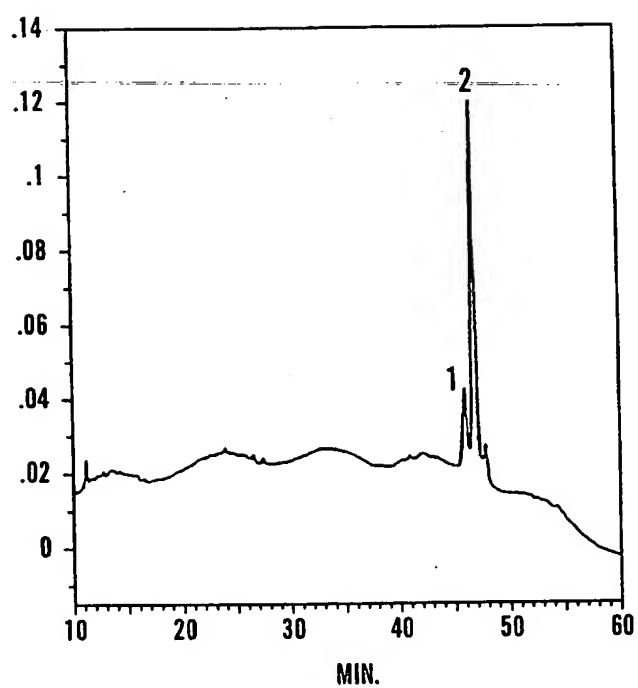
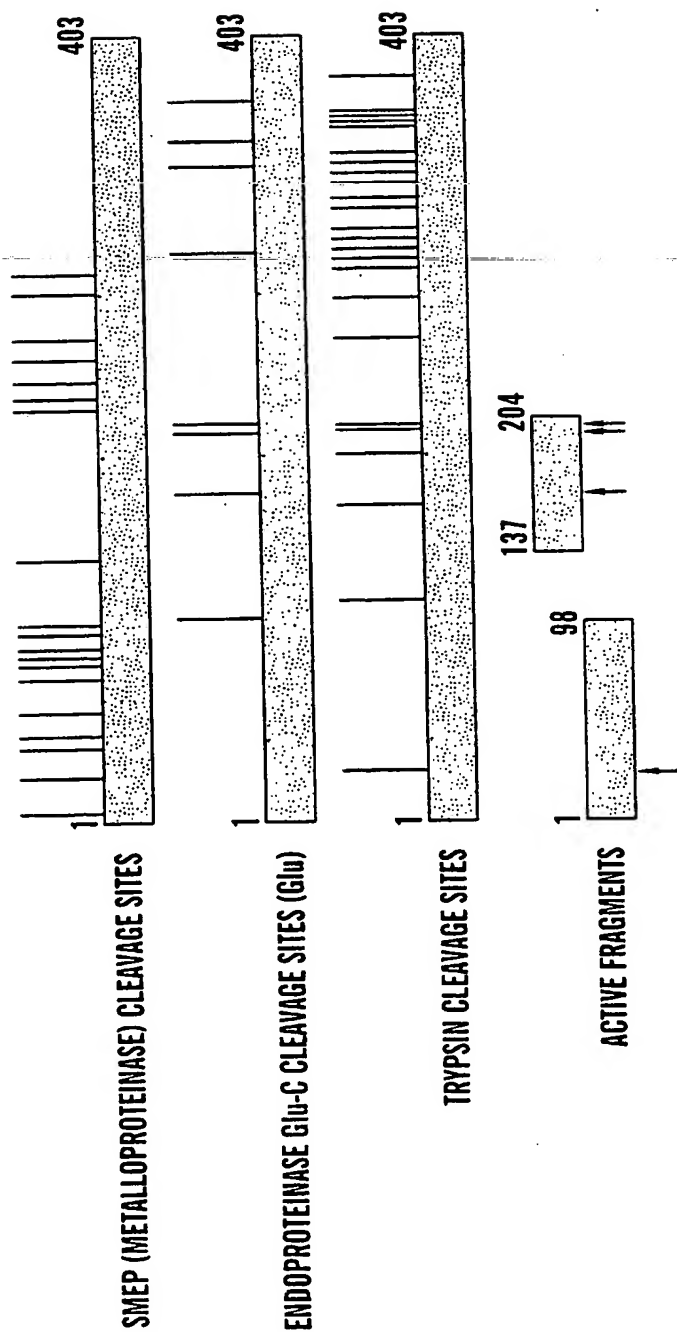


FIG. 5C

7/13

**FIG. 6**

8/13



↑ = CLEAVAGE AT THESE SITES ABOLISHES HR-ELICITING ACTIVITY OF HARPIN C305 AND OF PEPTIDES P64/P68 AND P91/P95

FIG. 7

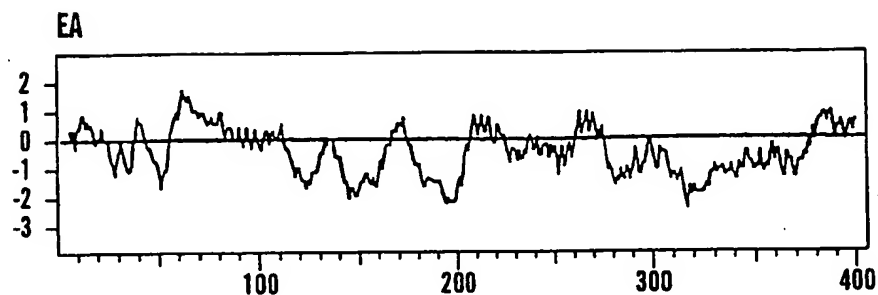
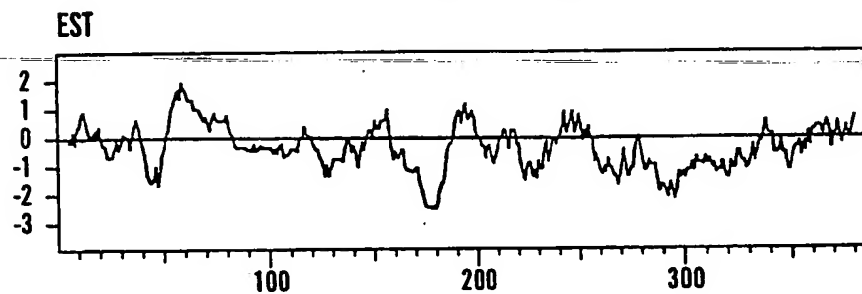
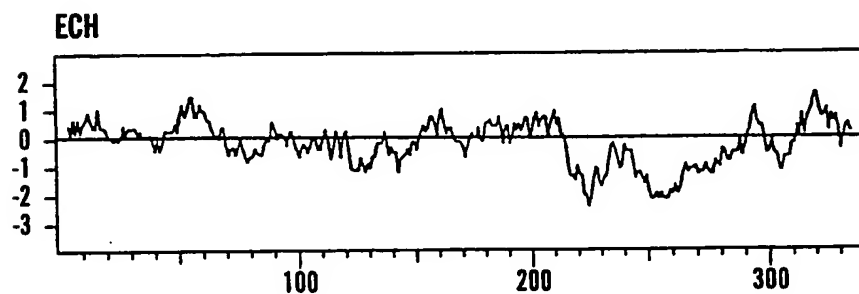
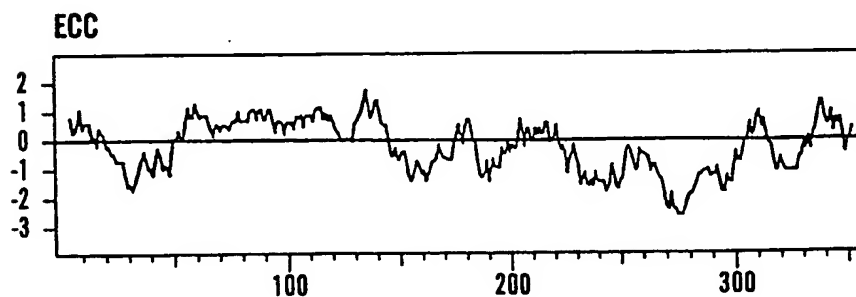
9/13

Gene	Organism ^a	N-terminal amino acid sequence
HrpN	Ech	MQITIKAHIGD <u>LG</u> V <u>SG</u> LGAQGLKGLNSAA
HrpN	Ecc	M.LNSLGGGA <u>S</u> .LQITIK.AGGNGGLFPSQ
HrpN	Eam	MSLNT <u>S</u> GLGA <u>ST</u> MQI <u>ST</u> CGAGGNNGLLGTS
WtsN	Est	MSMNT <u>S</u> PLGT <u>S</u> ALQVTL...GGNNGLMGTD
orf1B	Eam	MSILTLNNNTSS.SPGLFQSGGDNGLGCHA

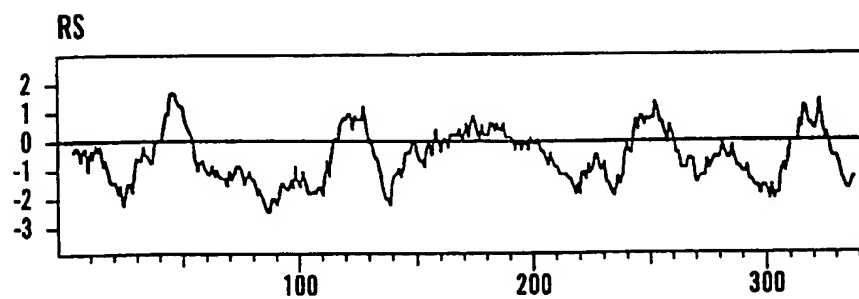
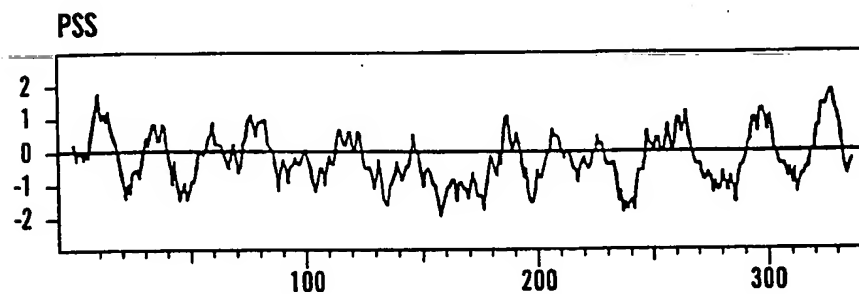
^aEch= *E. chrysanthemi*; Ecc= *E. carotovora* subsp. *carotovora*;
 Eam= *E. amylovora*; Est= *E. stewartii*.

FIG. 8

10/13

**FIG. 9A****FIG. 9B****FIG. 9C****FIG. 9D**

11/13

**FIG. 9E****FIG. 9F**

12/13

#1	HARPIN		403	HR
	1			+
#3	C-TERMINAL FRAGMENTS		403	+
#4	105		403	-
#5		169	403	-
#6		210	403	-
#7		267	403	-
		343	403	-
#8	N-TERMINAL FRAGMENTS			-
	1	75		+/-
#9	1	104		+
#10	1	168		+
#11	1	266		+
#12	1	342		+
#13	INTERNAL FRAGMENTS			+
#14	76	209		-
#15	76	168		+
#16	105	209		NA
#17		169 209		-
	105	168		
#18	99	209	HR	SYNTHESIZED OLIGOPEPTIDES
#19		137 204	+	150 179
#20		137 180	+	137 166
#21	105	180	+	121 150
#22		150 209	NA	137 156
#23		150 180	NA	

FIG. 10

13/13

N1; 5'-GGGAATTCATATGAGTCTGAATACAAGTGGG-3'
N76; 5'-GGGAATTCATATGGGCGGTGGCTTAGGCGGT-3'
N99; 5'-GGCATATGTCTGAACGCGCTGAACGATATG-3'
N105; 5'-GGGAATTCATATGTTAGGCGGTTCGCTGAAC-3'
N110; 5'-GGCATATGCTGAACACGCTGGGCTCGAAA-3'
N137; 5'-GGCATATGTCAACGTCCCAAACGACGAT-3'

N150; 5'-GGCATATGTCCACCTCAGACTCCAGCG-3'
N169; 5'-GGGAATTCATATGCAAAGCCTGTTTGGTGATGGG-3'
N210; 5'-GGGAATTCATATGGGTAATGGTCTGAGCAAG-3'
N267; 5'-GGGAATTCATATGAAAGCGGGCATTCAGGCG-3'
N343; 5'-GGGAATTCATATGACACCAGCCAGTATGGAGCAG-3'
C75; 5'-GCAAGCTTAACAGCCCACCACCGCCCATCAT-3'
C104; 5'-GCAAGCTTAAATCGTTCAGCGCGTTCGACAG-3'
C168; 5'-GCAAGCTTAAATATCTCGCTGAACATCTTCAGCAG-3'
C180; 5'-GCAAGCTTAAGGTGCCATCTTGCCCATCAC-3'
C204; 5'-GCAAGCTTAAATCAGTGACTCCTTTTTTATAGGC-3'
C209; 5'-GCAAGCTTAACAGGCCCGACAGCGCATCAGT-3'
C266; 5'-GCAAGCTTAAACCGATACCGGTACCCACGGC-3'
C342; 5'-GCAAGCTTAATCCGTCGTCATCTGGCTTGCTCAG-3'
C403; 5'-GCAAGCTTAAGCCGCGCCAGCTTG-3'

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10874

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/27 A01H1/00 A01H3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 96 39802 A (CORNELL RES FOUNDATION INC) 19 December 1996</p> <p>see abstract see page 10, line 30 - page 11, line 17 see page 12, line 21 - page 14, line 33 see page 17, line 33 - page 19, line 51 see page 27, line 11 - line 25 see page 29, line 28 - line 36 see examples 1-9 see figures 1,2</p> <p style="text-align: center;">--- -/--</p>	<p>1-3, 8-10, 15-28, 35-37</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

20 November 1998

Date of mailing of the international search report

28/12/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Panzica, G

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10874

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 612 848 A (SANDOZ AG ;SANDOZ LTD (CH); SANDOZ AG (DE)) 31 August 1994 see page 2, line 28 - line 35 see page 2, line 51 - line 54 see page 4, line 23 - line 30	1-3,8-10
A	see page 5, line 12 - line 18 ---	4-7, 11-43
X	WO 94 26782 A (CORNELL RES FOUNDATION INC) 24 November 1994 see page 2, line 11 - line 25	1-3, 8-10, 26-28
A	see page 3, line 14 - line 28 ---	4-7, 11-14
X	WO 94 01546 A (CORNELL RES FOUNDATION INC) 20 January 1994 see abstract see page 3, line 9 - page 4, line 10 see examples 1-15	1-3, 8-10, 15-28, 35-37
A	 ---	4-7, 11-14
A	WO 93 23532 A (RIBOZYME PHARM INC) 25 November 1993 see abstract see page 1, line 23 - page 2, line 8 see page 2, line 25 - page 3, line 5 ---	26-28, 35-37
X	WEI Z.M. ET AL.: "Harpin, elicitor of the hypersensitive response produced by the plant pathogen Erwinia amylovora" SCIENCE, vol. 257, 3 July 1992, pages 85-88, XP002085274 LANCASTER, PA US cited in the application see the whole document ---	1-3,8
X	BAUER D.W. ET AL.: "Erwinia chrysanthemi Harpin(Ech): an elicitor of the hypersensitive response that contributes to soft-rot pathogenesis" MOLECULAR PLANT-MICROBE INTERACTIONS, vol. 8, no. 4, 1995, pages 484-491, XP002085275 cited in the application see the whole document ---	1-3,8
A	 ---	4-7, 11-14
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10874

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEI Z.M., BEER S.V.: "hrpL activates Erwinia amylovora hrp gene transcription and is a member of the ECF subfamily of "sigma" factors" JOURNAL OF BACTERIOLOGY, vol. 177, no. 21, November 1995, pages 6201-6210, XP002085276	1-3,8
A	see abstract see figure 7 -----	4-7, 11-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/10874

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639802 A	19-12-1996	US 5650387 A	22-07-1997
		AU 5982196 A	30-12-1996
		CA 2223616 A	19-12-1996
		CN 1192647 A	09-09-1998
		EP 0871354 A	21-10-1998
		FI 974430 A	26-01-1998
		PL 323823 A	27-04-1998
		US 5776889 A	07-07-1998
EP 0612848 A	31-08-1994	AU 682674 B	16-10-1997
		AU 5631194 A	01-09-1994
		CA 2116202 A	25-08-1994
		CZ 9400404 A	12-07-1995
		HU 69614 A	28-09-1995
		JP 6319558 A	22-11-1994
		PL 302320 A	05-09-1994
		SK 20694 A	10-05-1995
		US 5550228 A	27-08-1996
		US 5552527 A	03-09-1996
		ZA 9401281 A	24-08-1995
WO 9426782 A	24-11-1994	US 5708139 A	13-01-1998
		EP 0702694 A	27-03-1996
		JP 8510127 T	29-10-1996
WO 9401546 A	20-01-1994	EP 0648266 A	19-04-1995
		JP 7509604 T	26-10-1995
WO 9323532 A	25-11-1993	AU 4234293 A	13-12-1993
		CA 2135643 A	25-11-1993
		EP 0641384 A	08-03-1995
		JP 7507931 T	07-09-1995
		MX 9302804 A	29-04-1994